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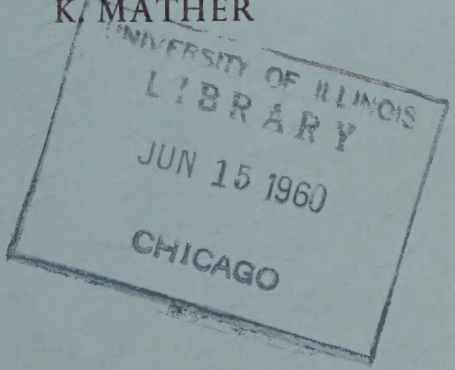

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BLACKWELL SCIENTIFIC PUBLICATIONS . OXFORD

The Heterotrophic Nutrition of *Chlorella vulgaris* (Brannon No. 1 Strain)

BY

D. J. GRIFFITHS, C. L. THRESHER, AND H. E. STREET

Department of Botany, University College, Swansea

With two Figures in the Text

ABSTRACT

A range of sugars, sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols have been tested as carbon sources for growth and as respiratory substrates using *Chlorella vulgaris*, Brannon 1, grown in darkness.

Much higher rates of growth and respiration were obtained with *d*-glucose than with any other substance tested. Ethanol (at 0·005 M.) sustained both growth and respiration at *c.* 50 per cent. of the level with glucose (0·028 M. or higher). Evidence was obtained that the organism can become 'adapted' to utilize *d*-galactose and sucrose as effective carbon sources. Sustained growth was not obtained with any of the other substances tested.

The glucose monophosphates, methanol and certain organic acids (oxalacetate, α-ketoglutarate, *cis*-aconitate, and pyruvate) clearly stimulated oxygen uptake but to a less extent than ethanol. The other substances tested were either inhibitory to respiration or inactive or of very low activity as substrates.

The growth in darkness and in liquid culture of *Chlorella* when supplied with *d*-glucose was insensitive to pH over the range 4·5 to 7·0 and was markedly enhanced by a high level of aeration. Gains in cellular dry weight ranging from 45 to 90 per cent. of the weight of *d*-glucose disappearing from the culture medium were recorded in growth experiments; measurements of CO_2 evolution in the Warburg indicated retention of up to two-thirds of the glucose-C in cell material.

INTRODUCTION

A NUMBER of workers have reported on the response of *Chlorella* to carbon compounds but very few of these investigations have been at all comprehensive or have involved the use of known species and strains. The present study was designed to define for a single strain of *C. vulgaris* the carbon compounds capable of maintaining its growth and aerobic respiration when cultured in darkness.

EXPERIMENTAL

General Experimental Procedures

The organism, *Chlorella vulgaris*, Brannon 1 (from the collection of Algae and Protozoa, Cambridge) was maintained on agar slants in diffuse daylight. The agar medium contained in g./l.: Proteose peptone (Difco), 1·0; KNO_3, 0·2; KH_2PO_4, 0·02; $MgSO_4.7H_2O$, 0·02; agar, 10·0. Subcultures from these slants were used to initiate stock cultures in a liquid culture medium.

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The inorganic constituents of the liquid medium were as Craig and Trelease (1937) modified by using ferric ethylenediamine tetraacetate (0·54 p.p.m. Fe) as iron source, by addition of molybdic acid (0·01 p.p.m. Mo) and by adjustment of the pH to 5·0. Stock cultures were maintained in darkness at 25° C. in liquid medium containing 1 per cent. (10 g./l.) *d*-glucose and sterilized by autoclaving (15 lb./in^2 for 10 min.).

The culture vessels were 250 ml. pyrex Erlenmeyer flasks containing 50 or 100 ml. medium. Stock cultures were repeatedly subcultured every 4 days by transfer of sufficient of the old culture to establish an initial population of 100 cells/mm.^{3}

Cells to initiate experimental cultures or for oxygen uptake experiments were harvested from 4-day-old stock cultures, washed by slow speed centrifuging (800 rev./min. for 30 min. at 10° C.) and suspended in inorganic liquid medium to give a density of 10^4 cells/mm.^{3} This suspension was added directly to the experimental medium to establish an initial population of 100 cells/mm.^{3} When studying oxygen uptake this suspension was shaken for 24 hr. at 25° C. in the dark to yield 'starved' cells. The withdrawal of samples for cell counting, the subculturing and the preparation of suspensions of washed cells were performed under aseptic conditions and in photographic safe-light (Ilford 907G—dark green). Experimental cultures, not less than 5 replicates per treatment, were incubated for 10 days, the cells being suspended once daily by gentle hand agitation. The progress of growth was followed by determination of the cells/mm.^{3} using a Burker ruled Haemocytometer slide and using the Spekker Absorptiometer (H760) with neutral filters and a calibration curve checked against haemocytometer counts. Cell dry weight was determined at the end of the growth period by drying the washed cells at 80° C. for 24 hours.

Total reducing sugar in the culture medium were determined by a modification of Wager's (1954) method (Ferguson, Street, and David, 1958*b*). Sucrose was hydrolysed by adding to 50 ml. medium, 10 ml. HCl (SG 1·1029) and incubating for 24 hours at 27° C.

Oxygen uptake was determined by the direct method of Warburg at 27° C. in an apparatus covered by a light-proof canopy so that only a low light intensity reached the flasks when reading the manometers. The shaking speed was 75 oscillations/min. and each flask contained 2 ml. of an inorganic liquid medium containing 5,000 washed cells/mm.^{3} Substrate dissolved in 0·5 ml. inorganic liquid medium was contained in the side arm and added 2 hours after equilibration. Oxygen uptake was followed for 22 hours and expressed as μl.O_2/10^7 cells/hr. based on the initial population.

Growth with various carbon sources

Other work in this laboratory (Ferguson, Street, and David, 1958*a*) has emphasized that the biological activity of certain sugars may be influenced by autoclaving them in the presence of the inorganic constituents of culture media. In such cases and whenever substances suspected of being thermo-

labile have been tested, they have been rendered sterile by treatment with anaesthetic ethyl ether ('etherized') and subsequently dissolved in sterile inorganic medium and distributed aseptically to sterile culture flasks.

Sugars and sugar alcohols have been tested at a concentration of 1 per cent. (Table 1). Clearly their activity is affected by autoclaving. This applies

TABLE 1

The Growth of Chlorella vulgaris, *Brannon 1, when Supplied with Sugars and Sugar Alcohols at a Concentration of 1 per cent. and Incubated in Darkness at 25° C. for 10 days*

Initial population 100 cells/mm.^{3}

Sugar or sugar alcohol	Population: cells/mm.^{3}	
	Complete medium autoclaved	Test substance 'etherized'
d-glucose	16,350±900*	16,400±880
d-galactose	6,033±940	3,666±230
d-fructose	4,875±450	2,730±130
d-mannose	3,400±80	3,590±330
d-xylose	3,280±90	3,125±160
l-arabinose	5,350±670	3,950±650
l-sorbose	2,550±70	2,140±50
l-rhamnose	3,800±450	2,950±150
d-ribose	3,180±120	—
α-methyl-*d*-glucoside	12,050±300	3,900±180
sucrose	11,760±820	7,480±180
maltose	11,000±920	3,450±240
lactose	6,590±400	2,340±200
raffinose	5,890±460	1,950±150
cellobiose	—	710±50
melibiose	—	800±50
mannitol	4,080±110	2,575±190
sorbitol	6,530±890	2,000±160
dulcitol	—	590±40
inorganic control	2,230±250	2,060±240

* Standard error of 5 replicate values.

particularly to the oligosaccharides and to the methyl-*d*-glucoside which undergo partial hydrolysis. *d*-glucose was the only substance which supported a consistently high rate of growth. The response to sucrose was variable. Chromatographic examination of the medium containing 'etherized' sucrose demonstrated its freedom from detectable monosaccharide contamination even after storage for 10 days at 25° C. Algal growth, in this medium was, however, associated with the accumulation of both fructose and glucose. In a separate series of experiments, using cells harvested after only a single 4-day passage in liquid medium, the growth in sucrose medium was either not significantly above the control or was significantly below the growth obtained when using cells harvested after repeated subculture in the dark in the liquid medium. Variable results were also obtained with *d*-galactose. Cell counts made at intervals during culture in *d*-galactose always showed a sharp rise in cell numbers towards the end of a 10-day growth period (Fig. 1). Values

of up to 10,000 cells/mm.^{3} were obtained in some instances with medium containing 1 per cent. 'etherized' *d*-galactose. These apparently 'adaptive' responses to sucrose and *d*-galactose are being further examined. The very small stimulations of growth resulting with monosaccharides (other than

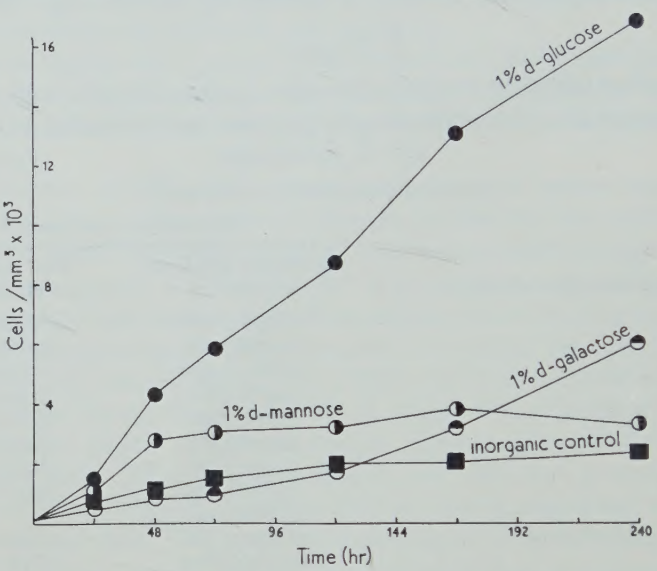

FIG. 1. Growth in darkness of *Chlorella vulgaris*, Brannon 1 supplied with various sugars as sole carbon sources.

d-glucose and *d*-galactose) and with maltose may result from contamination with traces of *d*-glucose for cell counts made at intervals during the 10-day growth period have usually shown an almost stationary population density from 72 hours onwards (Fig. 1 curve for *d*-mannose).

Sugar phosphates were 'etherized' as their sodium or potassium salts and used at a concentration equivalent to 1 per cent. of the free sugar phosphate (Table 2). Glucose-6-phosphate was inactive and the remainder quite inhibitory. A number of organic acids were also tested at a concentration equivalent in carbon content to 0·5 per cent. hexose (Table 2). Aqueous solutions of the acids were titrated to pH 5·0 with NaOH before addition to the inorganic liquid medium and autoclaving. Gluconic-δ-lactone and glucuronolactone were 'etherized' and tested at 1 per cent. concentrations. While most of the acids when tested under these conditions were inhibitory, a significant promotion of growth occurred with pyruvate, succinate, and fumarate. However, except with pyruvate, maximum population density was reached in 96 hours indicating that continuing growth is not achieved with these organic acids as sole carbon sources.

The finding that ethanol was an effective carbon source for the growth of both the Brannon 1 and the Pratt strains of *Chlorella vulgaris* (Street, Griffiths, Thresher, and Owens, 1958) led us to test a range of alcohols each over the

TABLE 2

The Growth of Chlorella vulgaris, *Brannon 1 when Supplied with Sugar Phosphates or Organic Acids and Incubated in Darkness at 25° C. for 10 days*

Initial cell population 100 cells/mm.^{3}

Substance	Population cells/mm.^{3}
At 1 per cent. concentration 'etherized'	
d-glucose	16,400±800*
glucose-1-phosphate	350±30
glucose-6-phosphate	1,430±50
mannose-6-phosphate	570±40
fructose-1-6-diphosphate	830±80
glucono-δ-lactone	360±30
glucuronolactone	350±40
inorganic control	2,060±240
Equivalent in C *content to 0·5 per cent. hexose*	
Whole medium autoclaved	
d-glucose	7,000±370
pyruvate	3,980±760
succinate	2,680±320
fumarate	2,580±440
cis-aconitate	1,800±190
β-glycerophosphate	1,735±190
acetate	400±80
formate	390±20
butyrate	366±40
propionate	290±40
citrate	283±60
inorganic control	1,730±140

* Standard error of 5 replicate values.

concentration range 0·1 to 0·0001 M. Methanol had a very low activity. None of the other alcohols had significant activity as carbon sources for growth and a number were inhibitory even at the lowest concentration tested (Table 3).

Oxygen uptake with various carbon sources

Despite the controlled prehistory of the cells, their rates of oxygen uptake in inorganic medium and in this medium containing 0·5 per cent. *d*-glucose varied from experiment to experiment. Mean values (calculated from 35 experiments) for the average rate of O_2 uptake per hour (averaged over 22 hours) for these two treatments were:

Inorganic control; 2·0±0·2 μl./10^7 cells
0·5 per cent. *d*-glucose; 41·2±1·4 μl./10^7 cells.

The enhancement of O_2 uptake resulting from the addition of 0·5 per cent. *d*-glucose was used as a reference standard in making comparisons between the activities of substances tested for their effects on O_2 uptake on separate

TABLE 3

The Growth of Chlorella vulgaris, *Brannon 1 when Supplied with Alcohols and Incubated in Darkness at 25° C. for 10 days*

Initial population 100 cells/mm.^{3}

Alcohol or control treatment	Optimum concentration tested	Population cells/mm.^{3}
d-glucose	0·056 M.	12,090±780*
inorganic control		1,850±60
ethanol	0·005 M.	6,080±300
methanol	0·025 M.	2,233±70
d-glucose	0·056 M.	14,200±860
inorganic control		2,270±164
n-propanol	0·0001 M.	1,475±170
iso-propanol	0·0001 M.	2,100±390
n-butanol	0·001 M.	2,990±200
tert.-butanol	0·1 M.	3,090±90
d-glucose	0·056 M.	13,800±900
inorganic control		1,890±230
n-pentanol	0·0001 M.	1,460±50
sec.-pentanol	0·0001 M.	1,740±240
tert.-pentanol	0·001 M.	2,240±130
d-glucose	0·056 M.	14,840±380
inorganic control		2,370±90
n-hexanol	0·0001 M.	1,940±210
n-heptanol	0·0001 M.	2,040±380
benzyl alcohol	0·0001 M.	790±40
ethylene glycol	0·001 M.	2,460±400

* Standard error of 5 replicate values.

occasions. The 'percentage activities' in Table 4 are calculated from the fraction:

$$\frac{\text{Highest average rate of } O_2 \text{ uptake obtained with the test substance} - \text{Average } O_2 \text{ uptake in inorganic control}}{\text{Average rate of } O_2 \text{ uptake with 0·5 per cent. } d\text{-glucose} - \text{Average } O_2 \text{ uptake in inorganic control}} \times 100$$

The low activities of sucrose and *d*-galactose are not at variance with the view that their utilization is an 'adaptive phenomenon'. The high activities of the glucose phosphates and of certain organic acids which are inactive in supporting sustained growth or even inhibitory, should be noted. The high activity of ethanol corresponds with its ability to function as an effective carbon source for growth.

The utilization of d-*glucose as a carbon source*

(i) *Aeration.* Evidence that, under the standard conditions of culture adopted above, aeration was a limiting factor was obtained when enhanced growth resulted from reducing the volume of medium from 100 to 50 ml. (10,480±460 as against 19,575±690 cells/mm.^{3} with 1 per cent. *d*-glucose). Using 50 ml. medium a further marked increase in final population density

TABLE 4

The Ability of Sugars, Sugar Alcohols, Sugar Phosphates, Organic Acids and Alcohols to Promote the O_2 *Uptake of 'Starved' Cells of* Chlorella vulgaris, *Brannon 1*

O_2 *uptake rate averaged over 22 hours*

Substance	Optimum concentration tested	'Percentage activity'
d-glucose	0·5% (0·028 M.)	100
glucose-6-phosphate	0·8%	49·5
glucose-1-phosphate	0·8%	28·7
d-galactose	1·0%	15·0
fructose-1-6-diphosphate	1·0%	12·3
d-mannose	1·0%	13·4
d-fructose	1·0%	11·6
maltose	0·5%	8·5
mannose-6-phosphate	0·4%	7·5
d-ribose	3·0%	6·5
l-arabinose	1·0%	6·1
sucrose	1·0%	4·5
sorbitol	0·5%	4·9
glucono-δ-lactone	0·5%	*
glucuronolactone	0·5%	*
oxalacetate	0·05 M.	32·0
α-ketoglutarate	0·01 M.	31·4
cis-aconitate	0·1 M.	22·9
pyruvate	0·1 M.	20·2
succinate	0·1 M.	6·7
malate	0·05 M.	4·0
citrate	0·1 M.	3·7
fumarate	0·005 M.	0·4
butyrate	0·005 M.	*
propionate	0·005 M.	*
ethanol	0·05 M.	54·0
methanol	0·1 M.	19·6
n-heptanol	0·001 M.	12·8
n-hexanol	0·001 M.	12·5
ethylene glycol	0·1 M.	2·7
n-propanol	0·05 M.	2·4
sec.-pentanol	0·01 M.	*
glycerol	0·01 M.	*
benzyl alcohol	0·1 M.	*

* O_2 uptake below that of the inorganic control.

resulted from shaking the cultures during each 24 hours for four-hourly periods on a Microid Silent Shaking Machine at 275 vibrations/min., each vibration involving a $\frac{1}{2}$-in. horizontal displacement of the culture flasks. The results of a specimen experiment are 23,200 for 'unshaken' as against 40,150 cells/mm.^{3} for 'shaken' cultures in 1 per cent. *d*-glucose. There was in 'shaken' culture a decreasing average cell weight until towards the end of the culture period and this corresponded with a rising absolute growth-rate (Table 5). With cell counts made every 24 hours it was, however, not possible to demonstrate

TABLE 5

The Changes in Growth-rate, Average Cell Weight, and Efficiency of Conversion of Glucose to Dry Weight in 'Shaken' Cultures in 1 per cent. d*-glucose Medium*

Initial population 100 cells/mm.^{3}

Growth period (hr.)	Increase in population density (cells/mm.^{3}) during period	Average dry weight per cell (mg.) at end of period	Dry weight increase / weight of glucose consumed
0–48	1,650	$7{\cdot}5 \times 10^{-8}$	0·45
48–96	8,550	$4{\cdot}4 \times 10^{-8}$	0·72
96–200	26,150	$2{\cdot}5 \times 10^{-8}$	0·90
200–240	3,900	$5{\cdot}7 \times 10^{-8}$	0·86

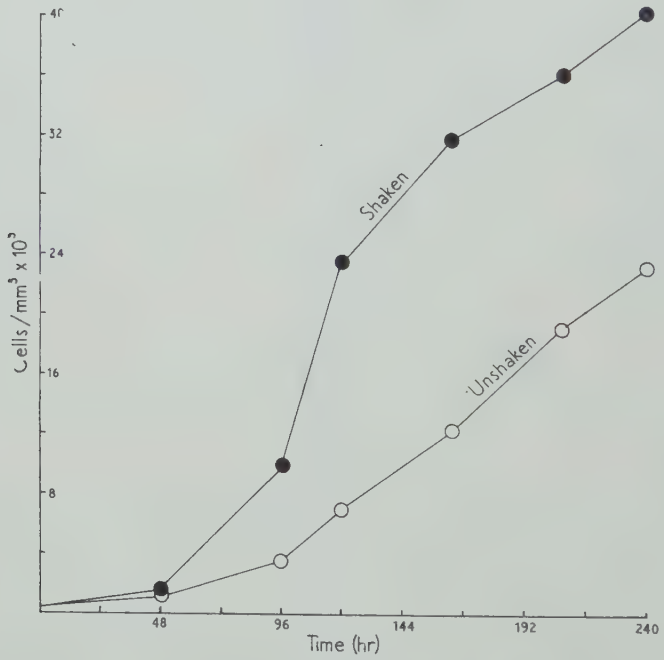

FIG. 2. The effect of 'shaking' on the growth of *Chlorella vulgaris*, Brannon 1 cultured in darkness and in medium containing 1 per cent. *d*-glucose.

any period of exponential growth. In 'unshaken' cultures no corresponding decrease in cell weight occurred and from 96 hours the absolute growth-rate remained linear (Fig. 2). The marked effect of aeration is further emphasized from the results of a separate experimental series using 50 ml. Quickfit tubes containing 20 ml. 1 per cent. *d*-glucose medium and continuously aerated with a fine stream of air at the rate of 50 ml. per min. During 10 days' growth at 25° C. under these conditions, the population density rose from 10 cells/mm.^{3} to 91,250 in the 'aerated' cultures and to 1,650 cells/mm.^{3} in parallel cultures not aerated.

(ii) *pH of the medium.* The effect of the pH of the medium was tested using 'unshaken' cultures initiated with cells harvested after a single 4-day liquid-culture period. The culture medium was sterilized by autoclaving at pH 5 and then adjusted aseptically to the various test pH values. The results using 1 per cent. *d*-glucose are shown in Table 6. A similar lack of sensitivity to initial pH over the range 4·5 to 7·0 was recorded using a limiting concentration (0·02 per cent.) of glucose.

TABLE 6

The Influence of the Initial pH of the Medium (1 per cent. d-glucose) on the Cell Density and Final pH after 10 days Growth in 'Unshaken' Culture

Initial population 100 cells/mm.^{3}

Initial pH	Final pH	Population cells/mm.^{3}
4·0	4·35	250
4·5	5·37	21,850
5·0	5·82	24,350
5·5	5·96	26,750
6·0	6·08	28,100
6·5	6·52	25,300
7·0	6·86	24,500
7·5	7·39	9,300

(iii) *Respiration.* Values for the RQ. of the endogenous respiration of 'starved' cells were within the range 1·1 to 1·4. With *d*-glucose supplied over the concentration range 0·02 to 1·0 per cent. the RQ. values were within the range 1·2 to 1·7. The RQ. was not significantly affected by glucose concentration and did not increase during the 22-hour test period indicating that its high value was not related to an oxygen deficiency.

Cells supplied with a low concentration of *d*-glucose will completely deplete the medium of sugar, and this can be effected during a Warburg run using 0·02 per cent. *d*-glucose medium. If it is assumed that the endogenous respiration is suppressed by exogenous glucose (Barker, 1936; Doudoroff, 1940) our results would indicate that the metabolism of 0·5 mg. glucose is associated with the absorption of not more than 100μl.O_2; a value consistent with incorporation of up to two-thirds of the glucose carbon into cellular material. This result is not inconsistent with the calculated values for the fraction: dry weight increase/weight of glucose consumed, quoted in Table 5.

DISCUSSION

The cells of *Chlorella vulgaris* (Brannon 1) can be maintained for long periods in the darkness with *d*-glucose as sole organic carbon source and they do not loose their green colour. Other workers using unicellular green algae have reported *d*-glucose to be superior to all other sugars tested (Bristol-Roach, 1926, 1927; Neish, 1951; Samejima and Myers, 1958). The impor-

tance, in testing oligosaccharides, of ensuring that glucose is not liberated during sterilization of the medium is emphasized by the results of the present study. In view of the earlier reports of galactose and sucrose utilization by unicellular algae, the indications now obtained that this may be an 'adaptive' process in *Chlorella* should be further investigated. The inactivity of the sugar phosphates as carbon sources for growth parallels the results of Samejima and Myers (1958) working with the Emerson strain of *C. pyrenoidosa*. This is now seen to contrast with the quite marked enhancement of O_2 uptake resulting from exogenous glucose monophosphates.

Our results, indicating the inability of organic acids to promote the continued growth of *Chlorella* in darkness despite the activity of some of the acids in promoting O_2 uptake, are in general agreement with those of Eny (1950, 1951). The general inactivity of alcohols as carbon sources and as respiratory substrates was to be expected, though the very high activity of ethanol (Street, Griffiths, Thresher, and Owens, 1958) particularly as a source of carbon for growth is regarded as a subject for detailed study.

The insensitivity of growth to pH, even under conditions of limiting glucose supply, suggests that if there is a specific carrier site for glucose at the cell surface, it must be protected by a buffering action of the cell wall.

The beneficial effect of mechanical shaking has previously been reported from culture studies with *Chlorella* (Pearsall and Bengry, 1940; Winokur, 1948). Pearsall and Loose (1937) and Pearsall and Bengry (1940) have also reported a decrease in average cell size during periods of rapid cell division in agitated cultures. How far this may be due to a more rapid release of autospores has not been ascertained. Clearly, provided the cultures are sufficiently aerated, a very rapid rate of growth in the dark can be maintained in a medium of inorganic salts and *d*-glucose.

High RQ. values in a nitrate-glucose medium have been reported by Warburg and Negelein (1920), Gaffron (1939), Pratt and Fong (1940), and Cramer and Myers (1948), for a range of algae. Pratt and Fong have also obtained RQ. values above unity with *C. vulgaris* and ammonium as the source of nitrogen.

Myers (1947) has represented the oxidative assimilation of glucose by *C. pyrenoidosa* by the equation:

$$C_6H_{12}O_6+O_2 = 5(CH_2O)+CO_2+H_2O$$

The oxidation of glucose by *Scenedesmus quadricauda*, according to Taylor (1950), goes to about 16 per cent. of completion, the remainder of the sugar being converted mainly to an acid-hydrolysable polysaccharide. Further understanding of these oxidative assimilations requires more detailed analysis on both a chemical and calorific basis.

Grateful acknowledgement is made to the D.S.I.R. for maintenance grants which enabled two of us (D. J. G. and C. L. T.) to take part in this work and to the Royal Society for the Spekker Absorptiometer.

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The Viability of Cereal Seed in Relation to Temperature and Moisture

BY

E. H. ROBERTS

(*West African Rice Research Station, Rokupr, Sierra Leone*)

With eight Figures in the Text

Abstract

Critical examination of previous work on the viability of cereal seeds shows that there is a simple mathematical relationship between temperature, moisture content, and period of viability. There is evidence that this relationship is similar for wheat, barley, and oats. If the moisture content and temperature of the seed is known it is possible to predict the expected life of the seed; or alternatively, it is possible to predict various combinations of storage conditions necessary to achieve a required period of viability. These results are discussed in relation to current theories concerned with factors which cause loss of viability in seeds.

Introduction

ONE of the problems facing the cereal breeder in the tropics is the difficulty of maintaining valuable breeding stocks of viable seed over a period of years. It is inconvenient having to sow rice every year in order to maintain seed stocks of breeding material when using ordinary storage methods. If the seed is not required every year this has several disadvantages, for in addition to wasting land and labour, the chances of losing purity both by mechanical mixture and natural cross-pollination are increased.

This paper deals with an investigation into the possibility of prolonging seed viability in storage. Very little new experimental work is described here, but what appear to be new interpretations are deduced from existing experimental data published by a number of workers. Further work on rice will be published later.

The literature on grain storage is large and the subject has recently been comprehensively reviewed in a symposium edited by Anderson and Alcock (1954). Much of the work has been carried out from the point of view of storing grain for food rather than for seed; similar problems are involved, but viability is not of direct importance in food storage except in as far as it indicates deterioration in food quality (Zeleny, 1954). Recent reviews concerned with the storage of seed—not necessarily cereals—for the maintenance of viability have been made by Crocker (1948), Porter (1949), and Owen (1956).

The number of investigations into rice viability which have been reported in the literature is small in relation to similar work on temperate cereal crops, but there is sufficient evidence to show that rice behaves similarly to

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the majority of other cereals. In cereals the main factors which affect viability are moisture and temperature: a decrease in either increases the longevity of the seed. It should be pointed out that this general rule does not apply to all plants: the seeds of some species, for instance citrus and coffee, loose viability more quickly if dried to low moisture contents (Porter, 1949; Owen, 1956). It is known then that for cereals a decrease in either moisture content or temperature increases the period of viability, but no general principle showing the relationship between these factors and viability has been described; therefore it has not been possible to predict the expected life for seeds stored under any particular set of conditions. The present paper deals with the evidence which suggests that such predictions can be made.

Experimental Evidence

Work on rice

Correlation of the results which have been published for rice is difficult for various reasons: in some of the work either the temperature or humidity (or moisture content) was not recorded because its importance was not realized; sometimes paddy (i.e. rice in the husk) was used and on other occasions the work was carried out on hulled rice. There is also the difficulty of defining criteria of moisture content, as different methods of measuring this give different results (Hlynka and Robinson, 1954); furthermore, many authors have not described which methods they used. Some variation between different authors' experimental results would therefore be expected even if the conditions of storage were said to be similar.

Figs. 1 and 2 show the relationship between moisture content and the time taken for the viability of rice seed to drop to two different levels of germination, 50 and 80 per cent. respectively. Viability is plotted on a logarithmic time scale. For the purposes of the present discussion, half-viability period is defined as the time taken from harvest to the point when germination has dropped to 50 per cent.; the time taken from harvest until germination has dropped to 80 per cent. is called the 80 per cent. viability period. Some authors have discussed the problem of viability in terms of the time taken for germination to drop to 0 per cent.; the usefulness of using this particular level of germination as a criterion of longevity, however, is limited because its position on the time scale can be altered to a very large extent by the behaviour of only a few seeds. The 50 per cent. level has been chosen as being the point which can be most accurately determined, and evidence is presented later which suggests that for cereals it represents a good estimate of the mean length of life of the seeds.

The 80 per cent. level has also been used in the case of rice because it happens to be the approximate level of germination which Saran (1945) found 7 years after paddy had been sun-dried after harvest to 3·6 and 4·5 per cent. moisture content and then stored in sealed containers. Germination tests at the end of this period were said to have given values of 'about 80 per cent.' This result of Saran's is important because it is the only one

concerning rice which has been found for such a long period of storage and for which moisture data is given. Unfortunately, no information was given about temperature of storage. The approximate temperature for the present discussion has been inferred from the average seasonal temperature given in the *Oxford Atlas* for the address of its laboratory.

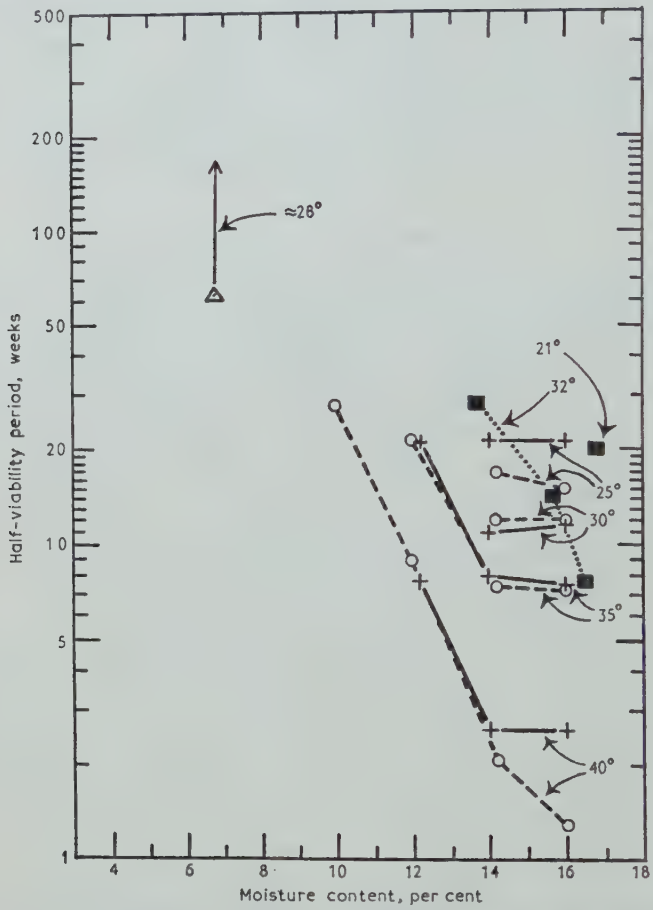

FIG. 1. The relationship between moisture content, temperature, and half-viability period in rice. Time is plotted on a log. scale. Temperatures are shown in degrees Centigrade. Data derived from results published by Kondo and Okamura (1930) for the varieties Kibiho (o----o) and Omachi (+——+), Dore (1955) (△), and Houston *et al.* (1957) (■·····■). For further explanation see text.

For the remainder of the results plotted in Figs. 1 and 2, the half-viability period and the 80 per cent. viability period were determined from the published data by plotting the curves of germination percentage against time; the points on the time scale at which the curves intersected the 50 and 80 per cent. levels of germination were then noted and the periods from harvest to these points were determined.

Dore (1955) who carried out his investigations using six different varieties

of rice compared the maintenance of viability under three conditions of storage—in the laboratory in paper bags, in sealed containers over anhydrous calcium chloride, and in an air-conditioned room (approximately 55 per cent. relative humidity and 68–78° F.). Viability was maintained best in the air-conditioned room, but by the end of the experimental period, which was

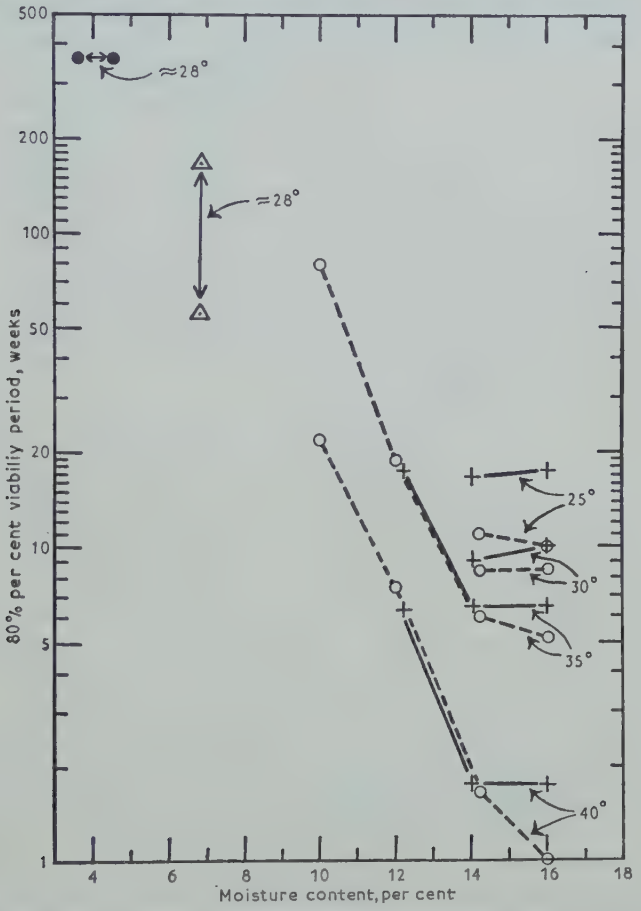

FIG. 2. The relationship between moisture content, temperature and 80 per cent. viability period in rice. Time is plotted on a log. scale. Temperatures are shown in degrees Centigrade. Data derived from results published by Kondo and Okamura (1930) for the varieties Kibiho (o-----o) and Omachi (+——+) Saran (1945) (●), and Dore (1955) (△). For further explanation see text.

a little over 3 years, viability had not dropped sufficiently for the results to be included in the present analysis. Viability was lost most quickly when the seed was stored in paper bags in the laboratory; these results cannot be used, however, because there is insufficient information about the conditions. The seed in the sealed containers was kept at laboratory temperature which was said to range from 78 to 88° F. The relative humidity in the sealed containers was said to be o per cent. However, this is unlikely because the

figure usually given for the absolute humidity of air over calcium chloride is 1·5 mg. water per litre of air at 30·5° C. (Vogel, 1948); the moisture content of saturated air at 30·5° C. is 30·9 gm. per cu. metre (Kaye and Laby, 1948); this gives a relative humidity of 4·85 per cent. This should be taken as a minimum figure because if calcium chloride of a higher moisture content were used, the relative humidity would be even greater. The difference in relative humidity between 0 and 4·85 although small is important, because a change in relative humidity at low values can cause a comparatively large change in the moisture content of cereal seeds, particularly on desorption (see Fig. 6). In Figs. 1 and 2, the value of 6·8 per cent. moisture content has been provisionally assigned to the data derived from Dore's results obtained for seed stored over calcium chloride, because this was the moisture content obtained in this laboratory in paddy which had been stored over calcium chloride for 23 months. Dore's results are represented in Fig. 1 by a vertical arrow which shows the spread of the values he obtained using six different varieties. The upper limit of the arrow is not known because the experiment was discontinued after 39 months when the germination of two varieties was still as high as 75 and 80 per cent.

Most of the points in Figs. 1 and 2 are derived from the results of Kondo and Okamura (1930) who used hulled rice of two varieties. The results for each variety have been plotted separately, one as a broken and the other as an unbroken line. Unfortunately there is an error of unknown magnitude in all these points because the date of harvest was not given. It was said to be '1927 seed' which presumably means that it was harvested in 1927. However, the seed was not put into the various storage treatments until May 1 1928, and this is the date from which the viability period has been calculated. The storage conditions from harvest until this time are not described; nevertheless, the factors which decrease viability must have been operating during the period before the storage experiment was started. It may be assumed then that the viability period represented by each point in Figs. 1 and 2 derived from the data of Kondo and Okamura is too small by a constant unknown amount. As the time scale is logarithmic, if a correction could be applied, this would have the effect of raising the curves and decreasing their gradients.

The remaining points in Fig. 1 are plotted from data derived from the results published by Houston *et al.* (1957).

It will be seen from an examination of Figs. 1 and 2 that there are some indications that at a given temperature the percentage moisture content is proportional to log. half-viability period or log. 80 per cent. viability period. The main features which disagree with this relationship are the inflections in the curves derived from the data of Kondo and Okamura; these indicate that above 14 per cent. moisture content an increase in moisture content does not cause a corresponding decrease in viability period. But by comparison with the curves derived from the data of Houston *et al.* and work on other cereals (see Figs. 3 and 5), these inflections in the curve would appear to be anomalous.

Work on cereals other than rice

It was possible to determine the half-viability points in the same way as was done for rice from the data of Robertson *et al.* (1939) for wheat, oats, and barley. In their investigations the dry seed (approximately 8 per cent. moisture content) was put into containers containing various salt solutions to give a range of relative humidities, and the containers were placed in a room maintained at a constant temperature of 21° C. Moisture contents

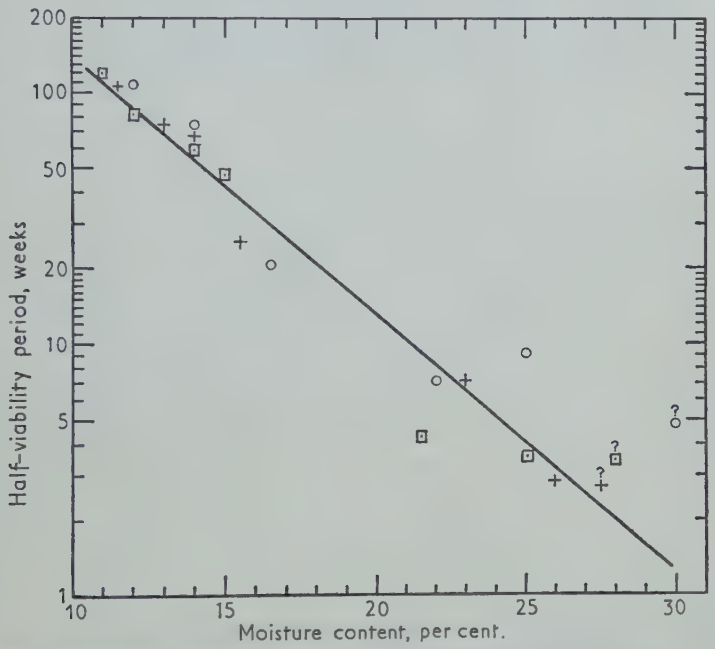

FIG. 3. The relationship between moisture content and half-viability period at 21° C. in wheat (+), oats (▣), and barley (○). Time is plotted on a log. scale. The regression line for wheat of log. half-viability period on moisture content is indicated and was calculated after omitting the doubtful value at 27·5 per cent. moisture content. Data derived from the results published by Robertson *et al.* (1939).

were determined at intervals, and the results show that equilibrium between moisture content and relative humidity was usually achieved within 2 to 3 weeks, but when the seed was placed in an atmosphere of 98 per cent. relative humidity, equilibrium was not reached before the viability had dropped to 50 per cent. For the purposes of plotting the values in Fig. 3, the moisture contents at 98 per cent. relative humidity have been assigned values which represent approximately the average conditions during the viability period. Because of this, the points representing moisture contents above 27 per cent. in Fig. 3 are not very reliable.

The half-viability period for wheat stored under various conditions has also been determined from the results published by Oxley and Hyde (1957). In this case, the published data are not sufficient to plot a curve of falling

viability against time; the figures available show the time taken until germination begins to fall and the time taken to the point when it reaches zero. Consequently for Oxley and Hyde's results, the half-viability period has been determined by assuming that the point of 50 per cent. germination occurred half-way in time between when germination began to fall and when it reached zero. Evidence, which is discussed later, that the frequency distribution of death points of stored cereal seeds is approximately normal supports the validity of this assumption. Oxley and Hyde's results are particularly

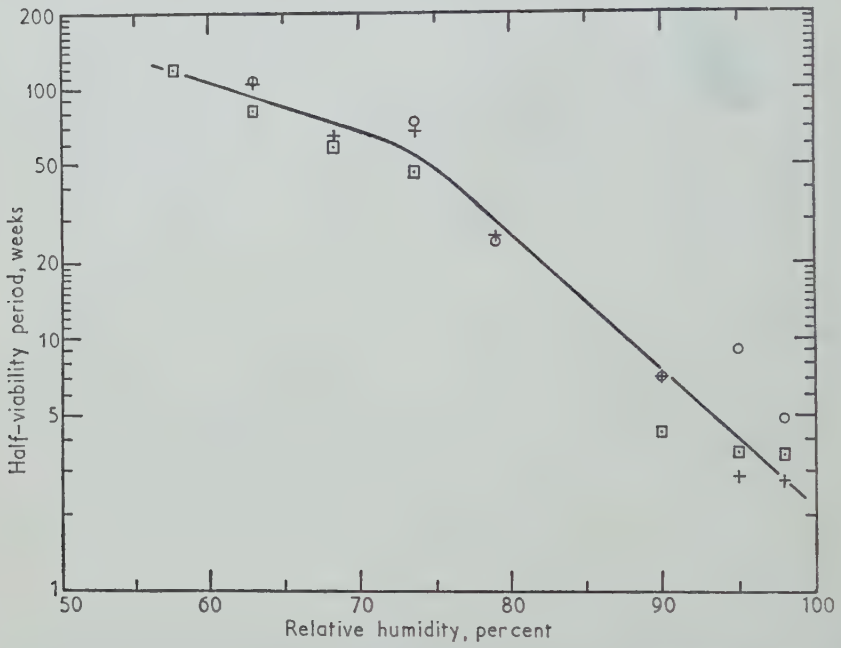

FIG. 4. The relationship between relative humidity of the inter-seed atmosphere and half-viability period at 21° C. in wheat (+), oats (▣), and barley (○). Time is plotted on a log. scale. Data derived from the results published by Robertson *et al.* (1939).

valuable because they represent the behaviour of wheat at two temperatures: the experiments were carried out at constant temperatures of 15° C. and 25° C.

The log. half-viability period for wheat, barley, and oats have been plotted against moisture content of the seeds (Figs. 3 and 5). An examination of these figures shows that for any given temperature there is good evidence for a straight-line relationship between moisture content of the seed and log. half-viability period. The curve shown in Fig. 3 which shows the regression of log. half-viability period on moisture content of seed has been calculated from the experimental data for wheat (excluding the point at 27·5 per cent. moisture which, as explained earlier, is of doubtful validity). It will be seen that this regression line also provides a good fit to the points obtained from

the experimental work on oats and barley. It therefore seems probable that these three cereals obey the same laws with regard to the maintenance of viability.

The data derived from Robertson *et al.* were used in Fig. 3 to plot log. half-viability period against moisture content. They also published sufficient data to make it possible to plot log. half-viability period against relative humidity of the inter-seed atmosphere. This has been done in Fig. 4 which shows that the relationship between relative humidity and log. half-viability period could also be considered as straight-line providing values above 74 per cent. are considered. But below this value, the relationship gradually changes, so that there is no simple relationship between relative humidity and half-viability period.

In considering why it is that moisture content gives a simple relationship with log. half-viability period, whereas relative humidity does not, it is necessary to consider the relationship between relative humidity and seed moisture content. Two curves are necessary to show this relationship because of the hysteresis effect: that is the equilibrium relationship is not the same on absorption of water as it is on desorption. Different investigators have obtained different results even when measuring equilibrium relationships on the same species at the same temperature. But in general the same type of result has been obtained, and the relationship is approximately the same for most cereals (Hlynka and Robinson, 1954). Two sets of curves for wheat at 25° C. have been plotted in Fig. 6, one set obtained by Babbit (1949) and the other by Hubbard *et al.* (1957). Alteration of temperature does not alter the shape of the curves, but slightly alters their position: an increase in temperature of 10° C. lowers the positions of the curves by about 1 per cent. moisture content in the middle ranges of the curves (Hubbard *et al.*, 1957). The data of Robertson *et al.* showing the relationship between relative humidity and moisture content of wheat on absorption at 21° C. have also been used to plot points in Fig. 6, and it is these results which should be primarily considered when discussing the relationship between viability and moisture illustrated in Figs. 3 and 4 because the relative humidity and moisture content values refer to the same samples of wheat as Robertson *et al.* used in their viability studies. On examining the equilibrium relationship between moisture content and relative humidity shown by the data of Robertson *et al.*, it will be seen that from between 74 and 78 per cent. relative humidity upwards the relationship can be considered as straight-line. From between 74 and 78 per cent. relative humidity downwards the relationship can also be considered as a straight-line, but of markedly different gradient. Now an examination of Fig. 4 shows that it is in the region between 74 and 78 per cent. relative humidity that there is an inflection in the curve showing the relationship between log. half-viability period and relative humidity. On the other hand, it was shown (Figs. 3 and 5) that the relationship between log. half-viability and moisture content is straight-line over all values. This is considered to be strong evidence that moisture content of the seed rather

than the relative humidity of the inter-seed atmosphere is the important moisture criterion in the control of seed viability.

If the curve representing the relationship between log. half-viability period and moisture content is a straight line, and an increase in moisture content results in a decrease in half-viability period, then it follows that for any given temperature this relationship may be expressed as

$$\log p = k_t - C_1 m, \qquad (1)$$

where p = half-viability period, m = moisture content, and k_t and C_1 are constants. The data shown in Fig. 5 strongly suggest that for different storage temperatures the gradients of the curves showing the relationship between log. half-viability period and moisture content are the same. This similarity of the curves at different temperatures is also suggested by the data for rice (Figs. 1 and 2) which is the only other data available for different storage temperatures. If the gradient is constant for all temperatures, it follows that C_1 in equation 1 is the same for all temperatures. However, the experimental results show that although the gradient does not alter, the position of the curve alters according to temperature. This means that the value of k_t is dependent on temperature.

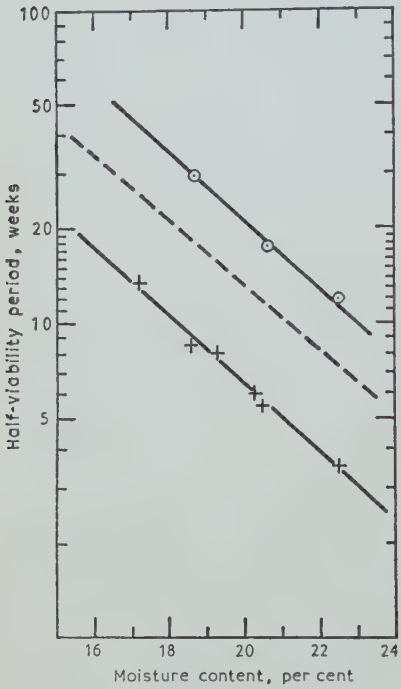

FIG. 5. The relationship between moisture content, temperature, and half-viability period in wheat. Time is plotted on a log. scale. The points were derived from the data published by Oxley and Hyde (1957). The regression of log. viability period on moisture content was calculated for the results at 25° C. (+); the line through the points representing 15° C. (◉) was drawn parallel to this. The broken regression line is the same as that shown in Fig. 3 and was calculated from the data derived from Robertson *et al.* (1939) for wheat stored at 21° C.

The relationship between temperature and viability is less obvious than that for moisture content and viability; this is because there are fewer experimental data available for different storage temperatures from which the differences in half-viability period due to temperature can be deduced. Nevertheless, all the available evidence also points to a similar relationship to that shown for moisture content and half-viability period. If log. half-viability period is inversely proportional to temperature, then the distances between the curves representing the different temperatures in Fig. 5 should be directly proportional to the temperature differences. The curve drawn through the points representing the half-viability periods for wheat stored at 25° C. is the regression of log. half-viability period on moisture content calculated from the points indicated, which were derived from the experimental

data. The curve representing the half-viability period at 15° C. has been drawn parallel to the regression line for 25° C. and it will be seen that it fits the experimental data well. The regression line calculated from the data on wheat stored at 21° C. derived from the work of Robertson *et al.* is also plotted in Fig. 5 and it will be seen that not only is the gradient of the regression line very similar to that calculated from the data derived from Oxley and Hyde, but it also occurs in approximately the expected place if log. half-viability period is proportional to temperature, i.e. it is roughly half-way between the lines representing 15° C. and 25° C. Any difference in gradient and departure from the theoretical position might be due to different methods of determining moisture content.

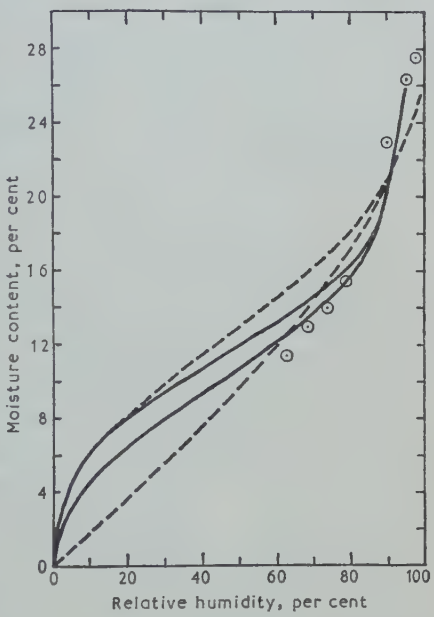

FIG. 6. Hysteresis curves showing the equilibrium relationship between moisture content of wheat grain and the relative humidity of the air. The curves by Hubbard *et al.* (1957) (——) and Babbit (1949) (-----) were obtained at a temperature of 25° C. The upper curve in each case shows the relationship on desorption and the lower curve the relationship on absorption. The points (◉) indicating data derived from Robertson *et al.* (1939) were obtained on desorption at 21° C.

If there is a simple negative correlation between temperature and log. half-viability period, then the equation describing this relationship will be of the same form as equation 1 which related moisture content to half-viability period. Therefore for any given moisture content the relationship between half-viability period and temperature may be expressed as

$$\log p = k_m - C_2 t, \qquad (2)$$

where t = temperature and k_m and C_2 are constants.

As the value for k_t in equation 1 varies with temperature, and as the expression $k_m - C_2 t$ in equation 2 shows the way in which temperature alters $\log p$, then it follows that k_t can be substituted by an expression of this form. The resulting equation which describes the relationship between half-viability period (p in weeks), temperature (t in °C), and moisture content (m as per cent.) therefore has the following form

$$\log p = K_v - C_1 m - C_2 t. \qquad (3)$$

By substituting values obtained from the regression lines calculated from experimental data it is possible to calculate values for the constants in equation 3. This has been done using data derived from Oxley and Hyde's experiments on wheat (Fig. 5) and the values obtained are $K_v = 4{\cdot}222$, $C_1 = 0{\cdot}108$, and $C_2 = 0{\cdot}050$. Using these values, equation 3 may be used

to predict the half-viability period of wheat and probably oats and barley as well under nearly all storage conditions. A graphical representation of equation 3 using these values is shown in Fig. 7. Obviously, not all storage conditions may be covered by the equation because, for instance, it is probable that there will come a point at higher temperatures where new factors, such as the heat destruction of thermolabile substances, will begin to operate and seed will be killed earlier than predicted by the equation. As yet there is

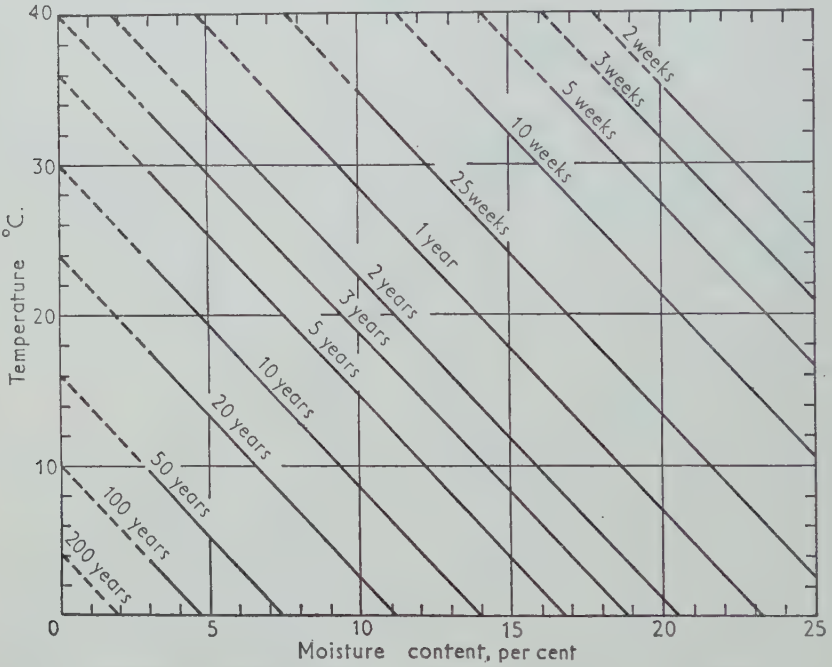

Fig. 7. The relationship between moisture content, temperature, and half-viability period in wheat using values of $K_v = 4{\cdot}2217$, $C_1 = 0{\cdot}1078$, and $C_2 = 0{\cdot}0501$ in equation 3. The half-viability isochrons are represented as broken lines above 35° C. and below 3 per cent. moisture content; this is because the same relationship may not obtain above 35° C. and moisture contents close to 0 per cent. might be impossible to obtain without damage to the seed.

little information on this point, but the data derived from Kondo and Okamura's work (Fig. 1) although variable, suggest that the space on the graph between the curves for 35° C. and 40° C. is larger than would be expected from the distances between the curves for the lower temperatures; this implies that at 40° C. loss of viability occurs earlier than would be expected from equation 3. It is suggested, therefore, that until further information is available, equation 3 should not be applied at temperatures above 35° C. However, this limitation should not be a practical disadvantage as it is not likely that any normal seed store would be maintained at such a high temperature.

The Q_{10} for decrease in half-viability period from the data shown in Fig. 5

is about 3·3. Hyde (1952) states that 'The rate at which seeds lose their viability increases with rising temperature. The temperature coefficient for this rate has been determined for several kinds of seeds [species not stated] and in each case the result was approximately 10. In other words the life-span of these seeds increased tenfold with each fall of 10° C.' Hyde's value for the Q_{10} for loss of seed viability is extremely high. A Q_{10} of 2 or 3 is normal for most chemical and enzyme reactions, and therefore the value determined in the present work would not be unexpected. It seems possible that Hyde's figure was determined for loss of viability at high temperatures where direct heat destruction of protein may take place. It is well known that the Q_{10} for this type of reaction can reach a very high figure, even up to 100.

The distribution of death points in time

So far, consideration has only been given to the points in time when the percentage germination of the seed drops to a certain level. The rate of fall of percentage germination has not been discussed. If a curve is constructed representing the percentage germination plotted against time, the result is a sigmoid cumulative frequency-distribution curve showing the frequency distribution of the death points of individual seeds. A large number of these curves has been examined for cereals both by plotting the published results of other workers and results obtained in this laboratory. This has been done in an effort to determine whether or not the distribution of death points is normal. If it is normal, it is necessarily symmetrical, and assuming that the original viability of the sample was 100 per cent., this means that the half-viability period is also the mean viability period of all the seeds in the sample.

One of the most convenient ways of dealing with large numbers of results when examining the closeness of fit of frequency distribution curves to the normal is to plot the cumulative frequency curves on a probability scale; when this is done, a normal distribution results in a straight line. This was the method used for examining the frequency distributions in the present investigations, and some samples of these curves showing the percentage germination of rice plotted on a probability scale against time are shown in Fig. 8; all the data used in this figure were obtained from Kondo and Okamura (1930). A large number of curves plotted in this way for a number of cereals have been examined and in general it has been found that the frequency distribution of death points is approximately normal. But in a few cases, particularly when rice is stored under conditions which lead to a more rapid loss of viability, it was found that viability dropped rather more quickly as time went on than would have been expected from a normal distribution of death points (see three of the curves in Fig. 8). This tendency shows that the distribution in these cases is slightly skew. However, even when the distribution is skew, the departure from the normal is not great, and therefore it is possible to estimate the half-viability point with a reasonable degree of accuracy by assuming that it occurs half-way in time between the point at which germination begins to fall and the point at which germination reaches

zero. The validity of using this method in the case of Oxley and Hyde's data (Fig. 5) is therefore substantiated.

If the distribution of death points is normal, then the time taken for viability to drop from any one germination level to another may be taken as a measure of the spread of this distribution. For example, using tables showing the area under a normal curve, it can be shown that the time taken

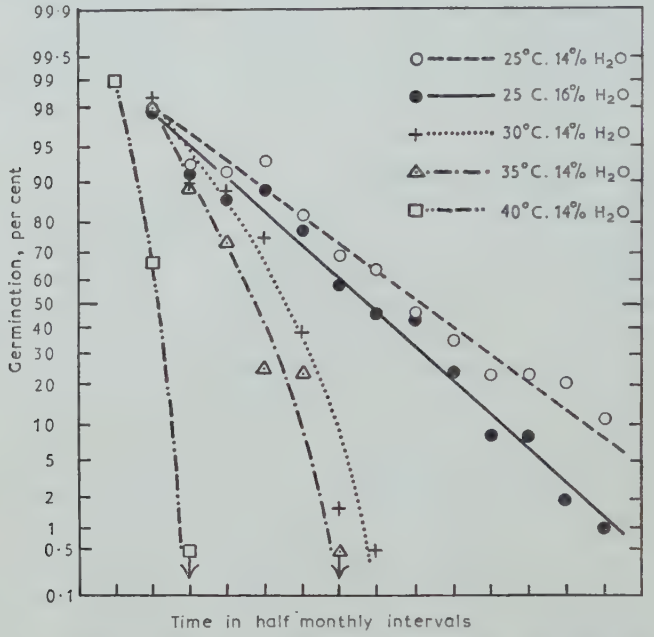

FIG. 8. Curves showing the rate of fall of viability in rice. Percentage germination is plotted on a probability scale. Because o cannot be shown on a probability scale, where a value of o per cent. was obtained experimentally, this is indicated on the graph by an arrow pointing downwards to a value less than 0·5 (200 seeds were used in each germination test). Data derived from Kondo and Okamura (1930).

for viability to drop from 80 to 50 per cent. germination represents 84 per cent. of the standard deviation (σ). There is evidence from Figs. 1 and 2 that the half-viability and the 80 per cent. viability curves are parallel. Because in these figures time is plotted on a log. scale, the period represented by the distance between these curves—and therefore σ—increases logarithmically with decrease in moisture content or temperature. As half-viability period also increases logarithmically in the same way, then σ is directly proportional to half-viability period. Therefore,

$$\sigma = K_\sigma p, \tag{4}$$

where K_σ is constant for a species. K_σ, then, can be calculated from observations on the time taken for viability to drop from any one germination level to another. By using K_σ in conjunction with equation 3 and tables showing

the area under a normal curve, it is possible to predict the time taken for viability to drop to any given level of germination.

Discussion

Theories concerned with the loss of seed viability

Various theories have been advanced as to the causes of loss of seed viability. These theories may be divided into two groups: those which postulate that loss of seed viability is due to some intrinsic factor of the seed's metabolism, and those which postulate that the causes are extrinsic to the seed and are due to other organisms which live in association with the seed, such as fungi and bacteria.

Examples of the first type of theory are as follows: Crocker (1948) suggested that intermediate products, accumulating as the result of anaerobic respiration, may be toxic to the seed. Oxley (1949) has suggested that the continued life of the seed depends on the use of some labile organic matter present in the embryo; as this substance becomes exhausted, the seed loses viability. D'Amato and Hoffman-Ostenhof (1956) have suggested that loss of viability may be due to some automutagenic action of the seed and have summarized the large volume of evidence concerning the increase in chromosomal and phenotypic aberrations in plants derived from populations of seed which have begun to lose viability. However, recent evidence suggests that this theory cannot be of universal application: Harrison and McLeash (1954) found that although lettuce seeds showed an increase in chromosome abnormalities with loss of viability, onion seed showed a low level of chromosome abnormalities at all levels of germination. D'Amato and Hoffman-Ostenhof (1956) have suggested that in some cases the loss of viability might be related to the inactivation of certain anaerobic dehydrogenase enzymes; this has been indicated by the use of oxygen-sensitive dyes such as tetraphenyltetrazolium chloride.

The second type of theory postulates that loss of viability is caused by organisms which live in association with stored seed, particularly fungi. The fungi may actually attack the seed, or their metabolic products may poison the seed, and it is possible of course that some of these metabolic products may have mutagenic activity. There is a large volume of work which demonstrates that decrease in seed viability is related to increase in activity of associated micro-organisms (Semenuik, 1954). Usually, however, it is difficult to distinguish which is cause and which is effect. Bottomley *et al.* (1950) showed that in yellow corn at relative humidities over 75 per cent. the mould count increases logarithmically with increase in relative humidity. The present results show that the half-viability period of wheat decreases logarithmically with increase in relative humidity above 75 per cent. It is interesting to note then that above 75 per cent. relative humidity there appears to be a direct linear correlation between mould count and half-viability period. Indirect evidence that fungi are one of the factors causing loss of viability in cereals has been produced by Tuite and Christensen (1955),

Golubchuk *et al.* (1956), and Papavizas and Christensen (1957, 1958). They showed that when wheat or barley seed is inoculated with fungi which are normally associated with grain in storage, the inoculated seed loses viability more quickly than non-inoculated seed.

Under conditions of temperature and moisture which lead to rapid deterioration in viability, it has been shown that values for seed respiration increase. Originally the assumption was made that this was entirely due to the respiration of the seed itself and therefore one might have supposed that the seed was using up its metabolites. But more recent work has indicated that the

TABLE 1

The Viability of Mouldy and Mould-free Wheat after Storage for 19 Days at 35° C. From Hummel et al. *(1954)*

Initial moisture content, per cent.	Condition of wheat	Viability of wheat after storage for 19 days, per cent.
14·9	Mould-free	70
	Mouldy	41
16	Mould-free	30
	Mouldy	10
18	Mould-free	8
	Mouldy	2
20·2, 24·2, 27·7, and 30·8	Mould-free	0
	Mouldy	0

greater part of this respiratory activity is due to associated micro-organisms (Milner and Geddes, 1954). In deciding whether or not micro-organisms are the factors causing loss of viability, it is not easy to carry out a critical experiment. One critical experiment would be to store sterile seed under various conditions and compare its retention of viability with non-sterile seed. Unfortunately, however, it is very difficult to obtain cereal seed which is entirely free from micro-organisms (Hyde, 1950; Christensen, 1951). A number of workers have shown that even in healthy, freshly harvested seed fungal mycelium is present between the cell layers of the pericarp or on the inner side of the pericarp (Christensen, 1951; Semenuik, 1954). However, Hummel *et al.* (1954) have found it possible to obtain a sample of wheat seed free from internal mycelium and it has therefore been possible to obtain seed which is probably sterile by surface sterilization of this sample. A study of sterile with non-sterile seed has made it possible to compare the relative changes brought about in respiration, viability, and chemical composition by storage fungi and the metabolic processes of the seed itself. This work has confirmed the view that most of the changes in respiration and chemical composition are brought about by fungi. The results on viability are of special interest. The figures obtained by Hummel *et al.* for wheat with an original viability of 95 per cent. stored for 19 days at 35° C. are given in Table 1.

It will be seen from an examination of this table that fungi have a deleterious effect on wheat viability at moisture contents of 14·9 to 18 per cent. However, it will also be noticed that at all these moisture contents, even the sterile seed loses viability very rapidly. At first sight the figures of 41 per cent. germination for mouldy and 70 per cent. for non-mouldy wheat at 14·9 per cent. moisture content suggest that mould is having a large effect; but when considered in terms of the time taken to reach 50 per cent. viability, it is obvious that the difference in days will not be great. Fungi therefore do have a definite deleterious effect at these high moisture contents, but from the practical point of view of maintaining viability their effect is not great. Unfortunately, there is no comparable information for storage at low moisture contents, but the information which is available suggests that at lower moisture contents the effects of fungi are even less important. Firstly, the correlation between loss of viability and fungal activity has only been shown at the higher humidities—in fact, the evidence indicates that storage micro-organisms are inactive at relative humidities below about 65 per cent. (Semenuik, 1954) and there is very little activity below about 75 per cent. (Milner and Geddes, 1954). Secondly, all the available evidence suggests that fungal activity is related to the relative humidity of the inter-seed atmosphere rather than the moisture content of the seeds themselves; this is shown by the fact that the marked increases in respiration due to microflora for different grains occur at an almost constant relative humidity of 75 per cent. in the inter-seed atmosphere, whereas at this relative humidity the equilibrium moisture contents of different grains may vary markedly (Milner and Geddes, 1954). The present investigations have shown that the important moisture consideration as far as seed viability is concerned is the moisture content of the seed rather than the relative humidity of the inter-seed atmosphere. These facts considered together suggest that fungal activity is not responsible for loss of viability at low moisture contents of the seed.

If fungi can play some part in causing loss of viability at high humidities but not at low humidities, this would explain why the frequency distribution of death points tends to deviate from the normal under conditions where viability is rapidly lost. Within the biological range of temperatures viability is only lost rapidly at high moisture contents. It follows that the relative humidity of the inter-seed atmosphere will be high and fungi will be active. As fungi have an effect on viability at high humidities and as it has been shown that inoculation of wheat or barley seed with storage fungi can increase the loss of viability (Golubchuk *et al.*, 1956; Papavizas and Christensen, 1957, 1958), it is probable that under conditions where fungi are flourishing and their numbers probably increasing exponentially, the expectation of the remaining live seeds will decrease more rapidly with time. Under these conditions one would expect a skew distribution in which the length of life of the seeds which die last is less than would be expected from a random distribution. If, however, the factors which are causing loss of viability are entirely intrinsic to the seed, as is suggested for lower moisture contents, one would

then expect the death points to be distributed at random around a mean value. The data in Fig. 8 fit this hypothesis.

It should be pointed out that in cases where micro-organisms are the immediate cause of loss of viability, it does not follow that they are the primary cause; this could still be an intrinsic factor. Went (1957) suggested that loss of viability might be connected with a decrease in resistance against fungi and saprophytic organisms. He showed that there are strong indications, at least in some species—e.g. *Godetia*, *Clarkia*, and *Boisduvillia*—that viable seeds produce antibiotics which are not present in non-viable seeds, and he suggested that loss of viability is largely due to the disappearance of antibiotics.

Practical implications

It is suggested that the present findings should be of help to those concerned with the design of cereal seed stores. In cases where small amounts of viable cereal seeds are required to be maintained over long periods at little expense, the most convenient method would be to dry the seed to low moisture content and place in air-tight containers; if necessary, the viability could be increased still further by placing the containers in a refrigerator.

When large amounts of seed and a large number of varieties have to be maintained over long periods, the most convenient method is to use an air-conditioned store. In this connexion it is hoped that equation 3 or Fig. 7 should be of help in designing air-conditioning equipment for any particular climate.

Future work

Further work is necessary to confirm the present findings and to discover the limits for the application of equation 3. The existence of probable upper temperature limits has been discussed. The maximum moisture contents of cereal seeds is probably about 30 per cent.; near this moisture content at higher temperatures germination takes place. As the distinction between physically and chemically bound water is not clear at very low moisture contents (Hlynka and Robinson, 1954), it may be impossible to obtain moisture contents close to 0 per cent. without damage to the seed. In Fig. 7 only temperature values as low as 0° C. have been indicated; this is because there is very little information about retention of viability at very low temperatures. However, there is no reason at this stage to suppose that equation 3 will not apply below 0° C. as the information which is available indicates that increase in viability may be expected on decreasing the temperature below 0° C. Sayre (1947) showed that when maize seed was stored at −17·8° C. either in sealed tubes or in the open air, it showed no indication of decreasing germinating capacity after 12 years. Even when the temperature of seeds is reduced to extremely low levels there is evidence that no damage to the seed

occurs: Lipman (1936) showed that when maize seeds were reduced to temperatures near the absolute zero for more than 42 hours, there was no decrease in viability. From this he concluded that seed could possibly be preserved indefinitely if kept at very low temperatures. If future work shows that equation 3 may be applied at all temperatures below 0° C. and at moisture contents close to 0 per cent., then the half-viability period would not be indefinite, and in fact would be capable of calculation. If the constant K_v in equation 3 is calculated for temperatures given in degrees absolute instead of degrees Centigrade, then K_v would be the same value as log. of the absolute or maximum half-viability period in weeks. (For wheat, the maximum half-viability period works out at about $1{\cdot}524 \times 10^{16}$ years, and therefore could be considered indefinite from the practical point of view.)

As yet, there is insufficient information to determine with confidence the constants in equation 3 applicable to rice, but the present analysis of the factors involved has provided a framework on which experimental work on rice is now being carried out.

Summary

1. Half-viability period is defined as the period from harvest until germination has dropped to 50 per cent. Evidence is presented which shows that half-viability period is dependent on the moisture content and temperature of cereal seed to which it bears a simple mathematical relationship. An equation is given whereby the expected half-viability period of wheat seed can be calculated; or conversely, it is possible to predict the various combinations of storage conditions to achieve a given half-viability period.

2. There is evidence that the equation which can be used to predict the half-viability period for wheat can also be used to predict the half-viability period for oats and barley.

3. Although the available evidence suggests that rice obeys similar laws to these other cereals, there is as yet insufficient experimental information to be able to predict the half-viability period in rice.

4. The distribution in time of the death points of individual seeds is discussed. Evidence is presented that in cereals this distribution is approximately normal except possibly in cases where viability is lost very rapidly when the distribution may become slightly skew. There is evidence that the spread of the distribution is directly proportional to half-viability period, and on the assumption that the distribution is normal, it would be possible to predict the percentage germination for any period under constant storage conditions.

5. These findings are discussed in relation to current theories concerned with explaining loss of seed viability. The view is put forward that although micro-organisms may slightly accelerate the loss of viability at high moisture contents, the main factors causing loss of viability in cereals are intrinsic to the seed.

Acknowledgements

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Leaf Development in *Narcissus pseudonarcissus* L.

II. The Comparative Development of Scale and Foliage Leaves

BY

M. P. DENNE[1]

(*University College, London*)

With twenty Figures in the Text

ABSTRACT

Details are given of the distribution of cell division and cell elongation in various tissues of the daffodil leaf.

The development of the vascular system is also described, and related to the intercalary growth of the leaf. The production of a new longitudinal vascular strand appears to be determined by the number of cells between the existing strands.

The scale and foliage leaves appear to originate from similar primordia. Their developments diverge when they are about 1 mm. long; a scale leaf is developed where most cell divisions occur in the sheath, and a foliage leaf is formed where there is a region of more rapid cell division at the base of the blade.

MATERIALS AND METHODS

A NUMBER of uniform bulbs of the variety 'Helios' was planted out in November 1954 and about twenty bulbs were dug up at monthly intervals until October 1955. The apices and younger leaves were fixed in chrom-acetic acid, and preserved in 70 per cent. methylated spirits. The older leaves were fixed and preserved in formalin acetic alcohol. The sections were stained in Heidenhein's Haematoxylin and Orange G.

The rate of cell division was determined along the longitudinal axis of the leaves. Longitudinal sections were cut in the median plane of the leaf, and each section was divided into a number of equal segments. In each segment the rate of cell division was determined separately in the central parenchyma tissue, the epidermis, and the palisade layers; the vascular tissue was omitted.

In the primordia less than 5 mm. long the rate of cell division was determined indirectly, using the formula:

$$\text{rate of cell division in segment} \propto \text{no. dividing nuclei} \times \frac{\text{mean cell length in segment}}{\text{length of segment}}.$$

In the primordia more than 5 mm. long, the rate of cell division was determined by counting the number of dividing nuclei per hundred nuclei, counting a thousand nuclei in each tissue of each segment of the leaf. Using either method it should be noted that it is the number of dividing nuclei that is being determined, this will be related to the true rate of cell division only if the

[1] Now at East Malling Research Station, Maidstone, Kent.

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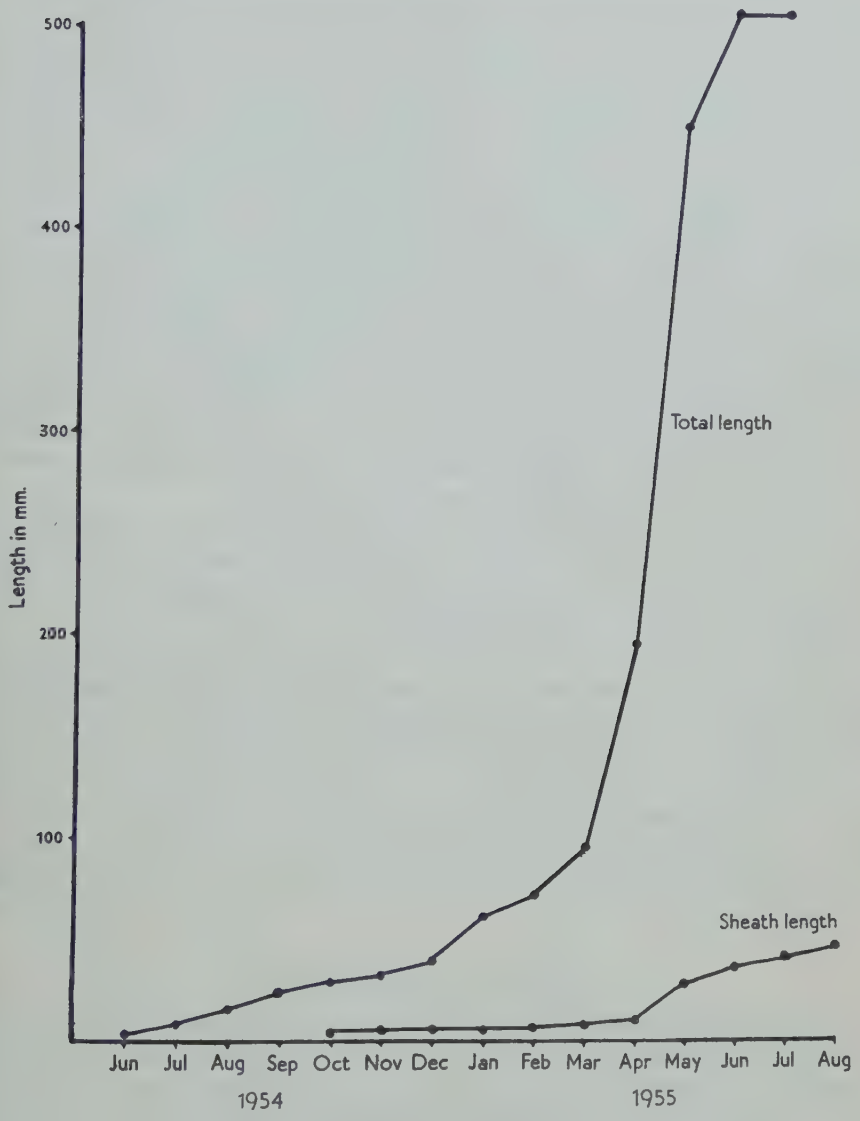


Fig. 1. Mean length of foliage leaves.

relative duration of interphase remains constant in different parts of the leaf. In Figs. 2 to 4 each point is the mean of at least five leaves, in Figs. 5 and 6 each point is the mean of two or more leaves.

The distribution of cell length was measured in the same sections. In each segment of each leaf 100 cells were measured at random in the epidermis, the palisade layers, and the central parenchyma tissue. Each point in Figs. 7 to 12 is the mean cell length measured from several leaves of a similar age.

The Form and Seasonal Cycle of the Daffodil Leaf

The mature foliage leaf consists of a basal sheath and a long flat blade. The sheath is usually tubular, and the blade is a prolongation of one side of the tube. In the mature scale leaf, the sheath is like that of a mature foliage leaf, but the blade is very short, and often colourless.

All the primordia are similar in external appearance until they are about 1 mm. long. By the time they are 3 mm. long there are obvious differences in the proportions of sheath and blade between scale and foliage leaves.

Fig. 1 shows the growth in length of foliage leaves from bulbs grown outside during 1954–5; each point is the mean length of all foliage leaves from bulbs dissected on that date. The tip of the blade emerged above ground level during January and February; at the end of April the blade was about half its final length, by June it was full grown, and in July it began to die back, eventually forming an abscission layer at the top of the sheath. These sheaths were packed with storage materials; they lived through the winter, and finally died in the following September or October.

The scale leaves grew very little during their first winter; rapid growth began in April, when the blade of the foliage leaf was about half grown. Throughout the next two winters these scales were packed with storage materials; they died in the following June or July.

In the bulbs grown outside in the season 1954–5, all the scale leaves on each apex were initiated during July and August 1954, one or more of the foliage leaves were initiated during September and the remainder in the following April or May. Between May and June 1955 the vegetative apex was transformed into a floral apex; the new main vegetative apex, which produced the main axis of the subsequent year's growth, developed in the axil of the penultimate leaf. The first few primordia produced by this new main apex usually became scales, but occasionally one or more foliage leaves were formed before the scales.

Initiation of the Primordia

Leaf initiation occurs on the side of the apex opposite to the point of origin of the previous leaf. The primordium slowly extends in both directions round the apex until it forms a complete ring, at the same time it lengthens, so the first-formed part of the ring is higher than the opposite part; the upper lip on the higher side develops into the blade, the basal ring becomes the sheath.

Leaf initiation is marked internally by periclinal divisions in all but the outermost layer of the tunica. The height of the young primordium is increased by further periclinal divisions, and continued cell divisions in this direction form long files of cells within the leaf. All growth in length of the leaf is due to the intercalary activity of these files of cells, as there is no active apical growth in the primordium. Growth in width and thickness of the leaf is due to occasional longitudinal cell divisions in the files of cells,

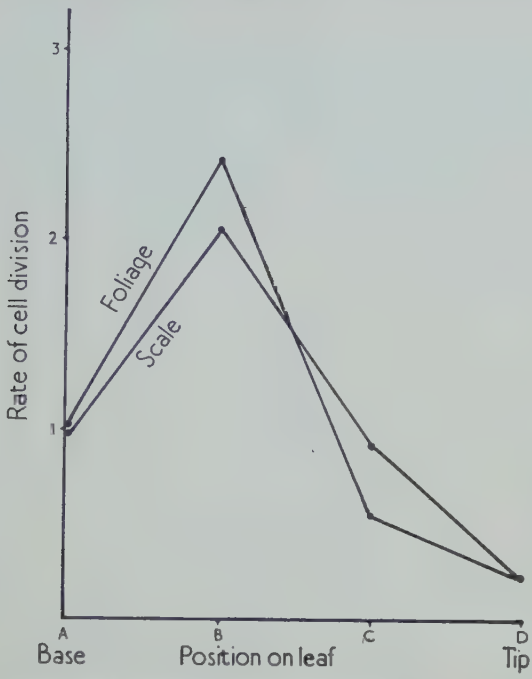


FIG. 2. The rates of cell division in scale and foliage leaves 0·5 to 1 mm. long.
Segment A was in the sheath.
Segments B, C, and D were in the blade.

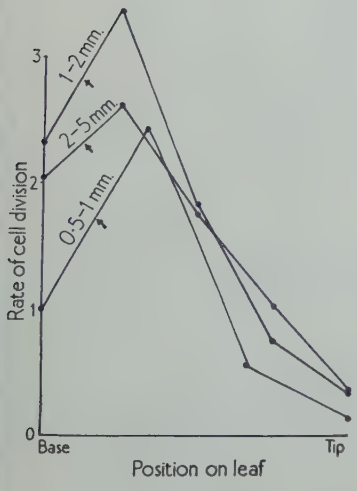


FIG. 3

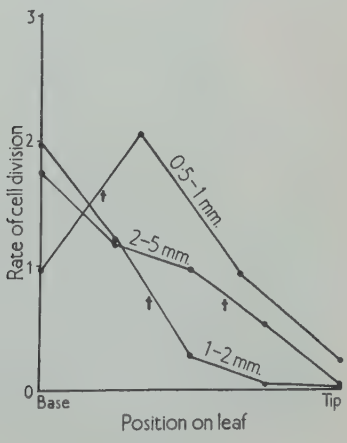


FIG. 4

FIG. 3. The rates of cell division in foliage leaves up to 5 mm. long.
FIG. 4. The rates of cell division in scale leaves up to 5 mm. long.
The arrows mark the approximate position of the base of the blade.

giving rise to new files after further transverse divisions. There is no distinct marginal growth, such as regularly occurs in the lamina of dicotyledons.

This early development of the daffodil leaf is similar to that reported in other monocotyledons, though apical and marginal growth have been reported in some monocotyledon primordia. Hsü (1944) described active apical growth in the cataphylls of *Sinocalamus*, and short-lived apical growth is reported to occur in the primordia of *Alstroemeria* (Priestley and Scott, 1937) and *Dactylis* (Bugnon, 1921). Sharman (1942) and Esau (1943) have described marginal growth in the leaf blade of *Zea*.

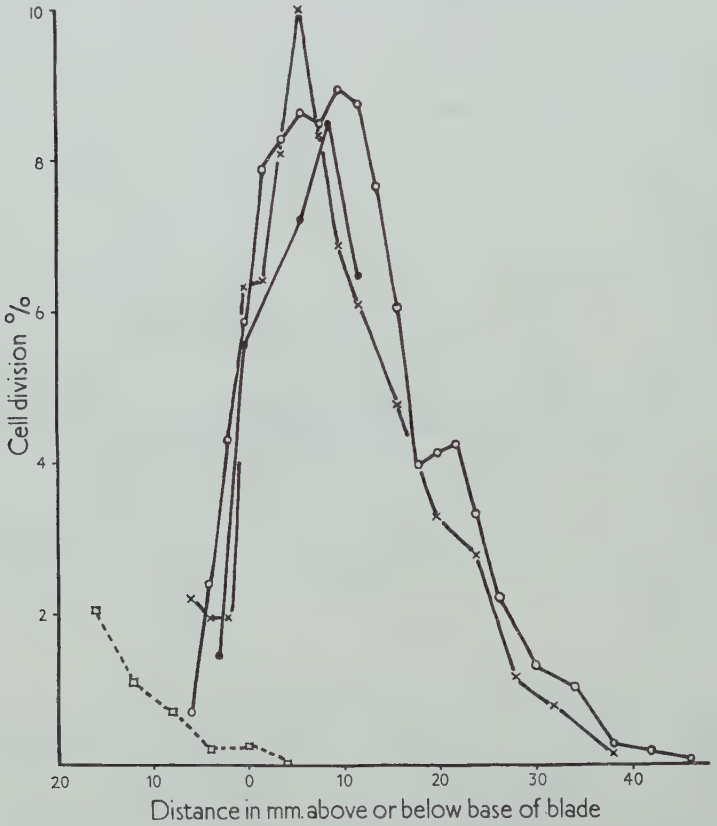


Fig. 5. The rates of cell division in palisade layers of foliage leaves more than 5 mm. long.
—●— about 15 mm. long.
—o— leaves of which the tip of the blade had just emerged from the bulb, about 100 mm. long.
—x— leaves of which the blade was about half its final length, about 250 mm. long.
---□--- leaves of which the blade was almost full grown, about 500 mm. long.

The Distribution of Cell Division

Leaves up to 5 mm. long. Fig. 2 shows the distribution of cell division in primordia 0·5 to 1 mm. long. This indicates a similar distribution of cell

division in all primordia of this length. The rate is faster at the base of the blade (segment B) than in the sheath (segment A).

Fig. 3 illustrates the distribution of cell division in foliage leaves up to 5 mm. long. There was a similar pattern of cell division in all these foliage leaves; the rate was faster at the base of the blade than in the sheath, i.e. an intercalary meristem occurred at the base of the blade.

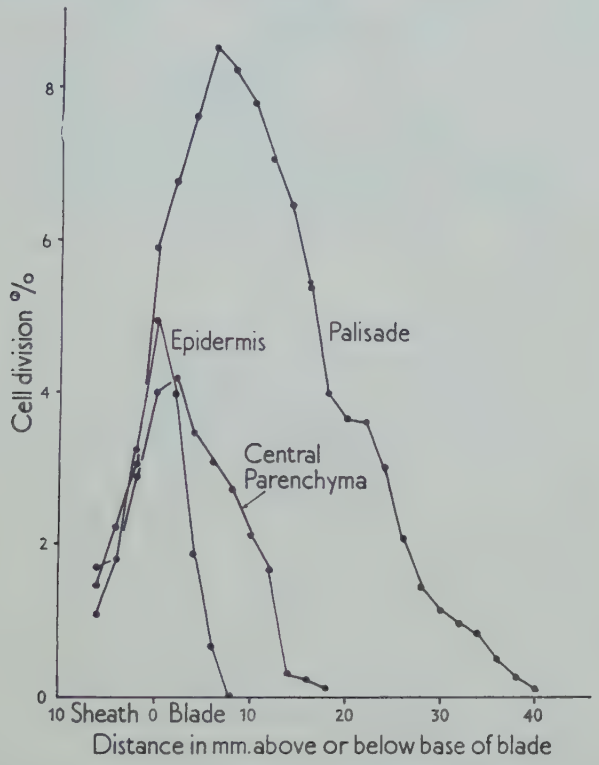
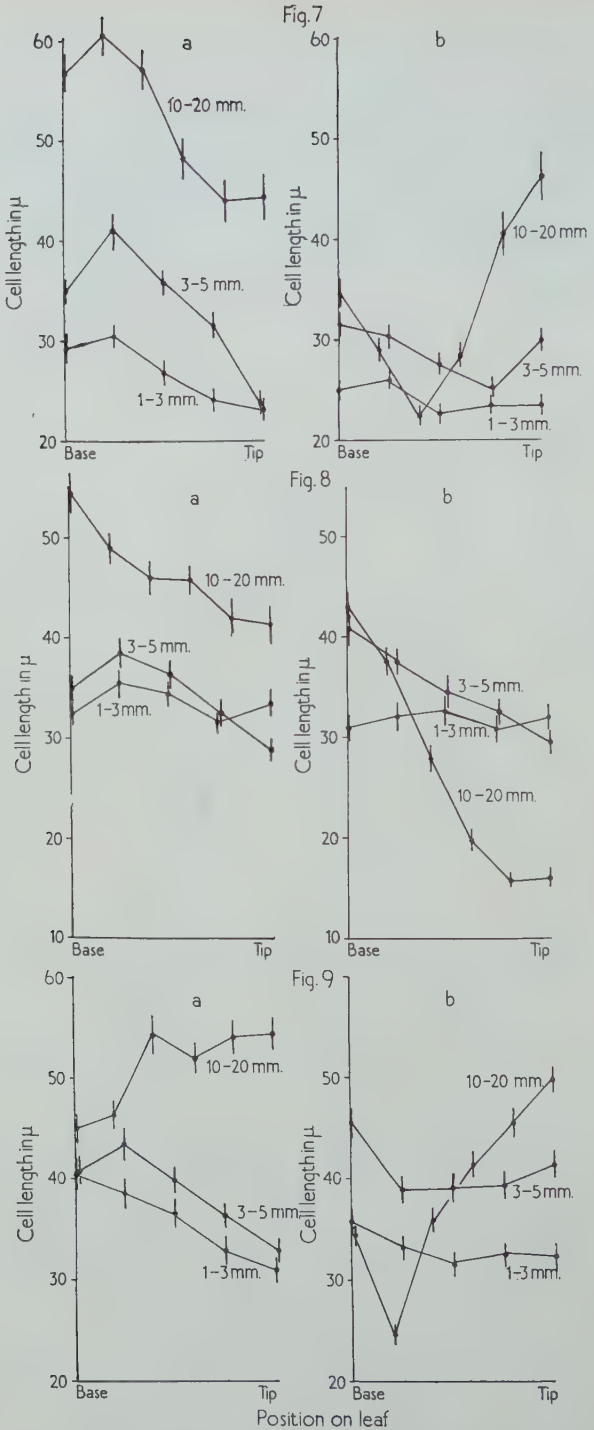


FIG. 6. Mean rates of cell division in different layers of foliage leaves during the period of rapid intercalary cell division.

Fig. 4 shows the distribution of cell division in scale leaves, which were comparable in length to those foliage leaves in Fig. 3. There was a marked change in the distribution of cell division between scales 0·5 to 1 mm. long and scales 1 to 2 mm. long, for all scales more than 1 mm. long had a decreasing rate of cell division from the base to the tip. At the base of the sheath the rate was about the same as that in the sheath of the foliage leaves, but there was no intercalary region of more active cell division.

Leaves more than 5 mm. long. Fig. 5 shows the distribution of cell division in the palisade tissue of older leaves, this suggests that there was a similar pattern of cell division in foliage leaves from about 15 mm. long until they were about half their final length; in all cases the rate of cell division was highest just above the base of the blade. There was also found to be a similar



FIGS. 7, 8, 9. Mean cell-length in the tissues of scale and foliage leaves. Vertical lines show twice the standard error on each side of the mean. *a*. Scale leaves. *b*. Foliage leaves.

FIG. 7, epidermal cells; FIG. 8, palisade cells; FIG. 9, central parenchyma.

distribution of cell division in the epidermis and central parenchyma of the same leaves. By the time the blade was about three-quarters of its final length all intercalary division had ceased. In the younger foliage leaves the rate of cell division decreased from the top to the base of the sheath, but cell division became more active at the base of the sheath after the leaf was about half its final length.

Fig. 6 compares the rate of cell division in different tissues of the foliage leaf during the period of rapid intercalary cell division. This shows that in the sheath and at the base of the blade the rate of cell division was similar in all three tissues. In the epidermis and in the central parenchyma the rate was highest at the base of the blade, but in the palisade layer cell division was more active about 5 to 10 mm. above this point. Epidermal cell division ceased about 10 mm. above the base of the blade (excluding stomatal divisions), the central cells continued to divide for about 15 to 20 mm. above the base of the blade, and the palisade until about 40 mm. above the base.

Cell Enlargement and Differentiation

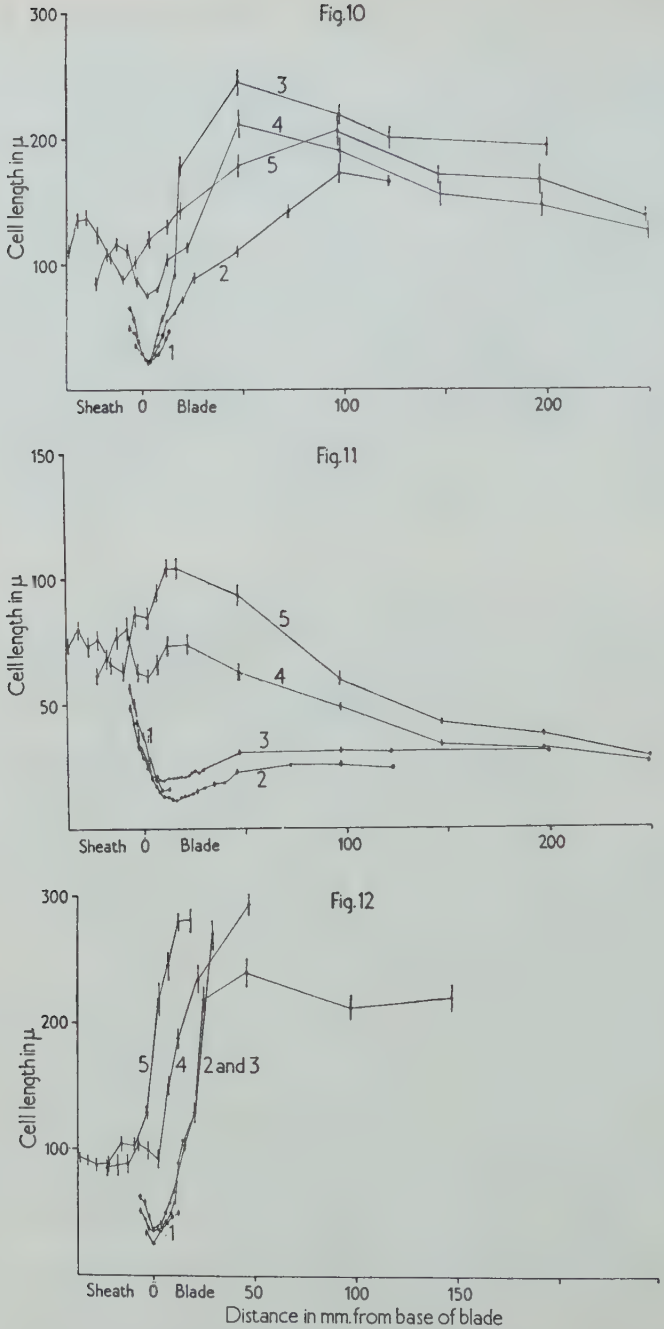
The cell lengths of foliage leaf primordia were not appreciably different from those of scale leaf primordia until the primordia were more than 3 mm. long; nor was there an appreciable difference between the cell lengths of primordia 1 to 2 mm. and 2 to 3 mm. long.

Figs. 7, 8, and 9 compare the cell lengths of scale and foliage leaves up to about 20 mm. long, while Figs. 10, 11, and 12 show the cell lengths of foliage leaves from about 15 mm. long until they were full grown. In general, the cell lengths of the younger foliage leaves increased in the sheath and at the tip of the blade, and increased to a lesser extent, or became shorter, at the base of the blade; and in scale leaves of similar lengths there was an increase in cell length throughout the length of the leaf.

There was a similar distribution of cell length in foliage leaves from about 15 mm. long until they were about half their final length, that is, during the period of intercalary cell division. By the time the leaves were about three-quarters of their final length cell division had ceased in the blade (as described above), so all further increase in the length of the blade must have been due to cell elongation at the base of the blade, as shown in Figs. 10, 11, and 12.

The sheath of the foliage leaf began to expand rapidly when the blade was more than half grown (Figs. 10, 11, 12). Cell elongation occurred throughout the sheath, but was greatest in the middle region.

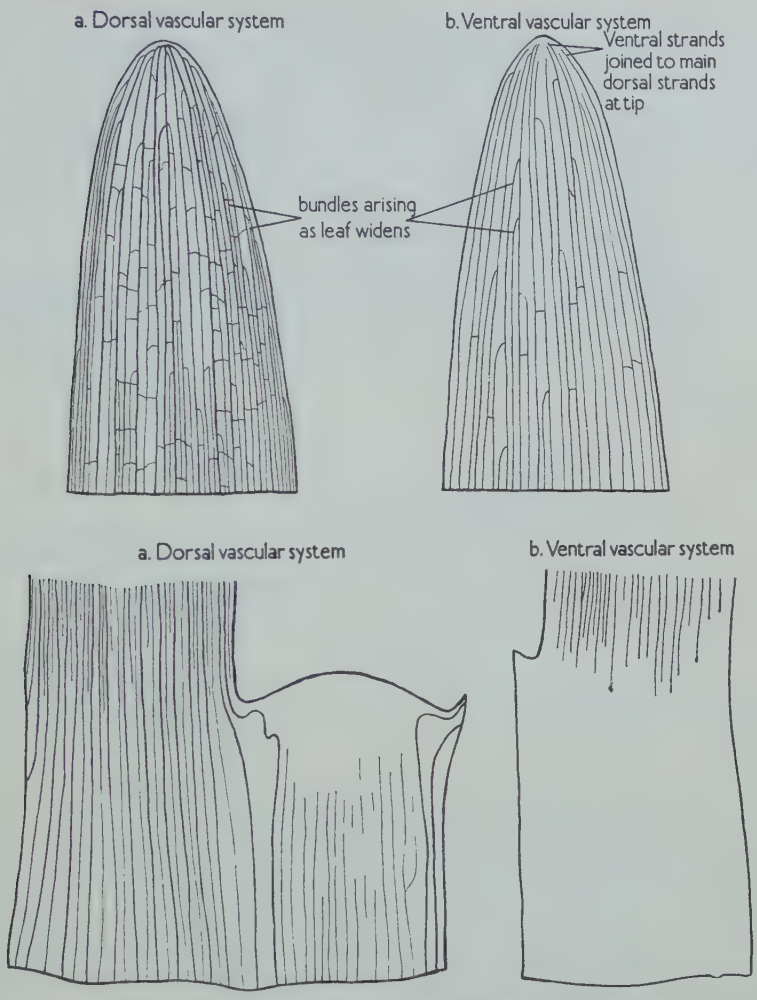
In the epidermal and palisade layers, cell elongation appeared to cease about 50 to 100 mm. above the base of the foliage leaf; this roughly corresponded to the point where the growing blade emerged from the surrounding sheaths of the bulb. The central cells were pulled apart below this point, leaving large cavities between the vascular bundles of the blade. This suggests that cell expansion ceased in the central parenchyma before it had finished in the other tissues.



FIGS. 10, 11, 12. Mean cell-length in the tissues of older foliage leaves. 1, leaves about 15 mm. long; 2, tip of the blade just emerged from the bulb; 3, blade about half its final length; 4, blade almost full grown; 5, blade full grown.

Vertical lines show twice the standard error on each side of the mean.

FIG. 10, epidermis; FIG. 11, palisade; FIG. 12, central parenchyma.



Figs. 13, 14. Vascular systems in the blade of a mature foliage leaf. Drawings of leaves cleared by boiling in lactic acid.

Fig. 13, leaf-tip; **Fig. 14,** the base of an almost mature foliage leaf; the blade was about full grown, the sheath was still expanding, the transverse interconnecting strands were not drawn.

The Vascular System of Daffodil Leaves

The mature foliage leaf. Most monocotyledon leaves have the 'parallel' type of venation; this consists of a number of strands running longitudinally through the blade and sheath into the stem, often interspersed with smaller longitudinal strands, which are usually restricted to the blade of the leaf. The daffodil leaf has two series of strands, a dorsal series arranged as described above, and a ventral series of minor bundles.

The dorsal series consists of a number of longitudinal bundles of varying

sizes, connected together by small transverse strands (Fig. 13*a*). The large longitudinal strands are here called the main dorsal bundles, and the smaller strands, which are regularly interspersed between the main bundles, are here called the subsidiary strands. The larger subsidiary bundles are called the secondary strands; one secondary strand lies between two main bundles. A series of smaller subsidiary strands, the tertiary strands, lie between the secondary strands and the main bundles, and there is often a set of still smaller bundles interspersed among the others.

The main dorsal and the secondary strands run through the blade and sheath into the stem (Fig. 14*a*). The main dorsal strands are joined together at the tip of the blade, and the secondary strands are connected to the main

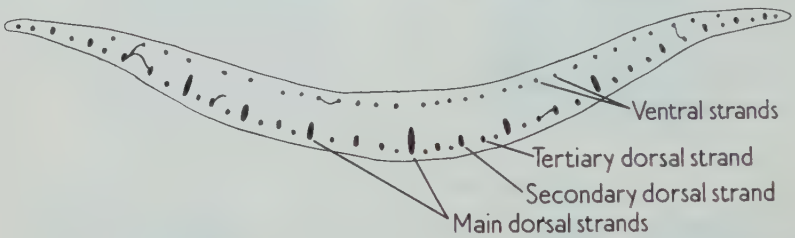


FIG. 15. Vein distribution in transverse section through the blade of a mature foliage leaf.

strands just below the tip (Fig. 13*a*). The smaller subsidiary strands are restricted to the blade; they are connected at both ends to larger dorsal strands.

The ventral series consists of a uniform series of small strands (Fig. 13*b*). These strands have the phloem orientated towards the ventral surface of the leaf, so are inverted with respect to the dorsal bundles. A few of the ventral strands extend from the tip of the blade to the upper part of the sheath; these are joined to the dorsal strands at both ends (Figs. 13*b*, 14*b*). All the other ventral strands are connected to neighbouring ventral strands at both ends. The ventral and dorsal parts of the vascular system are interconnected by numerous small transverse strands (Fig. 15).

In the part of the sheath opposite to the blade, the vascular system consists only of a few dorsal strands, containing single tracheids.

The total number of strands in each series varies with the width of the blade. Passing from the tip to the base of the leaf, the width of the blade increases, then decreases slightly and as it does so the number of strands increases and decreases.

The mature scale leaf. As in the blade of the foliage leaf, the dorsal and ventral vascular systems are present in the rudimentary blade of the scale.

The development of the procambial system. The first procambial strand of the primordium becomes visible in the apex at about the same time as the initiation of the primordium begins; this strand later becomes the median dorsal strand of the leaf. As the primordium extends round the apex the lateral main dorsal strands develop. The cells of these procambial strands

have dense contents; since they are the same length as the surrounding cells at first, the strands can be distinguished only when the surrounding cells vacuolate (Fig. 16). These main strands cannot be said to develop acropetally or basipetally, but are blocked out as a whole by the vacuolation of the surrounding tissues; they are always in continuity with strands in the stem, and joined at their tips above (Fig. 17).

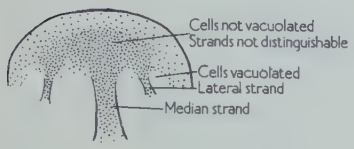


FIG. 16

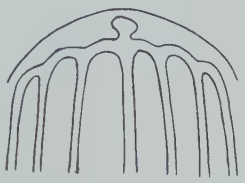


FIG. 17

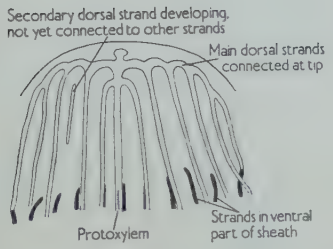


FIG. 18

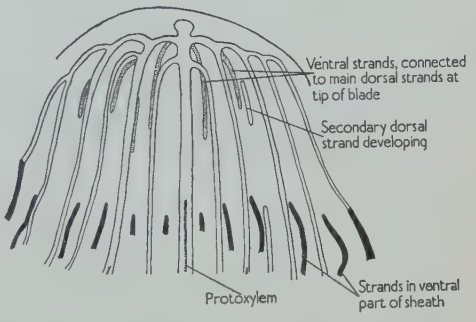


FIG. 19

FIG. 16. Primordium showing the development of the median and the first pair of lateral dorsal strands. Drawing reconstructed from longitudinal sections.

FIG. 17. Primordium showing the continuous system of main dorsal strands. The ventral and subsidiary dorsal strands had not yet developed.

FIG. 18. Procambial system of a primordium about 0·75 mm. long. The secondary dorsal strands were developing; the ventral strands had not appeared. Drawing reconstructed from tangential longitudinal sections through the primordium.

FIG. 19. Procambial system through a primordium about 1 mm. long. Drawing reconstructed from tangential longitudinal sections through the primordium.

Meanwhile the young primordium slowly increases in width, due to occasional longitudinal cell divisions which occur throughout the leaf. Consequently the number of cells between two procambial strands increases; there appears to be a definite limit to the number of cells occurring between two strands, and before this limit is exceeded a subsidiary strand is formed between the two existing ones. This is illustrated in Fig. 20 which shows that a new bundle is formed when the two existing strands were more than eleven cells apart. Near the tip of the leaf drawn in Fig. 20 there was no sign of a subsidiary strand between the two main strands which were eleven cells apart, lower in the blade there were twelve cells between the main strands, and here a subsidiary strand was visible; at the top of the sheath of this leaf one less cell division had occurred between these two main strands, and the subsidiary

strand had not formed, while at the base of the sheath the strand appeared once more. This relationship was found in a number of leaves.

At first the subsidiary strands are not connected to the rest of the system (Fig. 18), but later they become joined to a neighbouring strand at the tip, and extend through the sheath into the stem (Fig. 19). The primordium is about 600 μ to 800 μ long by this time.

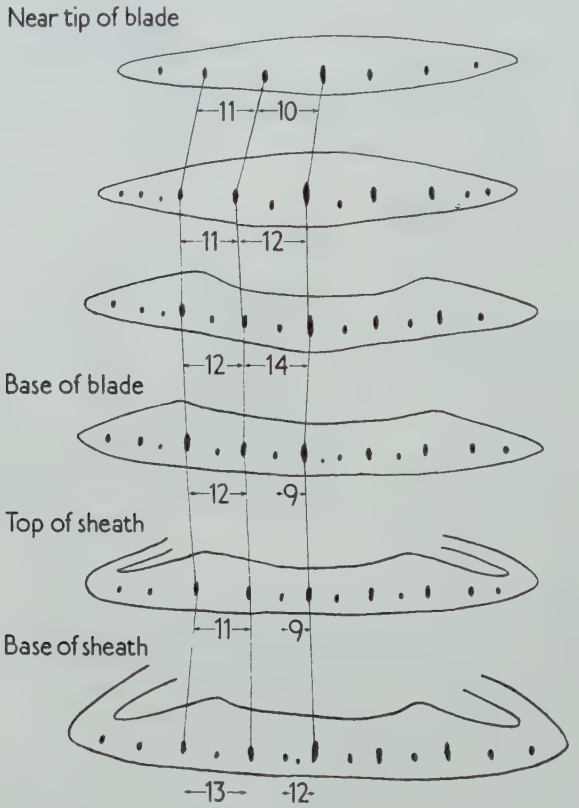


Fig. 20. Transverse sections through a primordium about 1 mm. long. The dorsal vascular system is drawn, the ventral system has been omitted.

Up to this stage procambial development is identical in presumptive scale and foliage leaves. Soon after, the growth of the scale leaves is restricted to the sheath region, while the foliage leaves have a region of rapid intercalary growth at the base of the blade. In the foliage leaves the width of the blade continues to increase slowly in this region of rapid intercalary growth; as this happens the number of cells between the main and secondary strands increases, and tertiary strands are developed in the same way as the secondary. These later formed strands do not develop towards the tip of the blade, and rarely penetrate far into the sheath, since the development of the strands occurs only in the region of active cell division. When the blade is more than half its final length the rate of cell division slows down, consequently the width

of the newly formed part of the blade decreases slightly, the number of cells between the strands decreases, and some of the smaller strands rejoin neighbouring bundles. When intercalary cell division ceases entirely, all the remaining tertiary and small subsidiary bundles rejoin the larger strands at the top of the sheath.

The ventral strands originate soon after the secondary dorsal strands are formed, just before the divergent growth of the scale and foliage leaf primordia (Fig. 19). In the foliage-leaf primordia their development is similar to that of the smaller subsidiary strands.

The differentiation of the xylem. The protoxylem develops acropetally in the main dorsal strands; it appears first in the median main strand (Fig. 18), then in the lateral main strands towards the margin of the leaf (Fig. 19), and in the larger subsidiary strands. The protoxylem of the larger dorsal bundles is always continuous through the intercalary growing region, as new tracheids appear to be differentiated from procambium at about the same rate as the old ones are destroyed by stretching.

By the time the leaf is about 50 mm. long the protoxylem has reached the tip of the median main bundle. Following this, there is a basipetal wave of protoxylem differentiation, then a basipetal wave of metaxylem development.

When the leaf is about half its final length, the basipetally developing strands have differentiated as far as the junction of sheath and blade, i.e. they have differentiated through the intercalary region.

Discussion of Vascular Development

The development of the vascular system of a number of other monocotyledon leaves has been described elsewhere, and in general it resembles that reported here for the daffodil. Priestley, Scott, and Gillett (1935), working with *Alstroemeria*, state that the main procambial strands are blocked out as a continuous system, and the subsidiary strands develop basipetally; this is very similar to the development in the daffodil. But Sharman (1942), working with *Zea*, and Pray (1955) investigating *Hosta*, report that the main strands develop acropetally.

Several authors have stated that the number of subsidiary strands depends on the growth in width of the intercalary meristem (Bugnon, 1921; Ertl, 1932; Chouard, 1931). Bugnon says that a new strand is formed in *Dactylis* when the distance between two older strands reaches a maximum, but in the daffodil it seems that the number of cells between the strands is more important than the actual distance. The discontinuity of the newly formed subsidiary strands, described above, suggests that it is the number of cells which determines the production of the strand, not the production of the strand which influences the amount of cell division between the strands.

Trécul (1878) described the differentiation of xylem in the leaves of the Gramineae; he reported acropetal differentiation in the main bundles, followed by general basipetal development. This is similar to the course of develop-

ment described in the daffodil, and in many other monocotyledons, for instance, in *Tradescantia* (Scott and Priestley, 1925) and in *Zea* (Sharman, 1942). Sharman states that the protoxylem is collapsed in the region of intercalary growth, implying that the water supply to the blade must pass through or between living cells, but in the daffodil the xylem was found to be continuous in this region, and is also reported to be continuous in *Cladium* by Conway (1936) and in *Zea* by Esau (1943).

Summary of the Intercalary Development of the Daffodil Leaf

The scale and foliage leaves of the daffodil are similar in external appearance until they are about 1 mm. long, and until this time they are also similar in the distribution of cell division and cell length, and in the development of the vascular system. Later their growth becomes localized; where active cell division is restricted to the base of the leaf a scale is formed, and where cell division occurs in the sheath, but there is an intercalary region of more rapid cell division in the blade, a foliage leaf is developed.

Active intercalary division continues until the leaf is about half its final length. In the basal 5 mm. of the blade the cells divide at about the same rate in all tissues, and the cells are about the same length in all tissues. In this region protoxylem is present in the main dorsal and larger subsidiary dorsal strands.

Just above this region, about 5 to 40 mm. above the base of the blade, the rate of cell division is faster in the palisade layers than in the epidermis or in the central parenchyma cells, and hence the palisade cells are shorter than those of the other tissues. In this region the protoxylem develops in all but the smallest vascular strands.

Cell elongation ceases in all tissues above about 50 mm. from the base of the blade. At this level metaxylem is differentiated in all the vascular strands.

Intercalary cell division slows down when the leaf is more than half its final length. Further increase in leaf length is due to cell elongation at the base of the blade.

Acknowledgements

The writer's grateful thanks are due to Dr. D. J. B. White for encouragement and guidance throughout, to Professor W. H. Pearsall for much helpful advice, and to the Department of Scientific and Industrial Research for a maintenance grant. The material in this paper is part of a Ph.D. thesis submitted to the University of London.

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Swede (*Brassica napus*) seeds with split cotyledons

I. The distribution of split cotyledons on plants

BY

T. P. PALMER

Crop Research Division, Department of Scientific and Industrial Research, Christchurch, New Zealand

Abstract

Plants of a line of swedes which gave a high proportion of split cotyledons were self pollinated at intervals of 6 to 8 days. Different plants, and different pollinations on the same plant, produced different proportions of affected cotyledons. Thus the tendency to produce split cotyledons is inherited, and also affected by environmental variations. In another experiment, plants were sprayed with various concentrations of tri-iodobenzoic acid, indoleacetic acid, and 2·4-dichlorophenoxyacetic acid. These treatments were not shown to affect the proportion of split cotyledons, nor was any variation with time detected. When both cotyledons were split, they tended to be affected to the same degree.

Introduction

SEVERAL authors have recorded differences in the proportions of seeds with divided cotyledons from different fruits on a plant (Straub, 1948; Haskell, 1954; Palmer, 1957). Haccius (1955) showed that various growth substances induced cotyledon abnormalities, including splitting, in *Eranthis hiemalis*.

In some lines of swedes (*Brassica napus*) up to 70 per cent. of the seeds may have divided cotyledons. An analysis of the effect on this character of some growth substances, of position on the plant, and of time of development of the seeds was undertaken.

Material and Methods

Two experiments were undertaken, in 1954 and 1956.

1954 Experiment. One Crimson King swede plant was self pollinated in 1953, giving nineteen viable seeds. Seventeen of these produced normal seedlings; the other two had divided cotyledons. The plants from these two and five of the others were self pollinated at intervals of 6 to 8 days in 1954. Seedlings from these pollinations were classified as schizocotyledonous or normals.

1956 Experiment. Eleven plants from lines of swedes known to give a high proportion of split cotyledons, and twelve plants from lines giving a low proportion, were sprayed at weekly intervals from the beginning of flowering with solutions of 2·4-dichlorophenoxyacetic acid (1, 10, and 100 p.p.m.), indoleacetic acid (1 and 10 p.p.m.), and tri-iodobenzoic acid (10, 100, and 1,000 p.p.m.). At intervals of 3 or 4 days, a date tag was tied

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round the highest opened flower on each stem. Each siliqua was harvested separately, and its position on the plant, and the approximate flowering date of the flower from which it developed were recorded. Each seedling which grew from these siliquas was examined, and the degree of splitting of each cotyledon was recorded on a scale ranging from '0' to '3', '0' being normal, and '3' resulted from a complete splitting of the cotyledon, giving in effect two cotyledons in the place of one. Cotyledons scored '2' had most of the cotyledon blade, but none of the petiole, divided, while '1' was any effect between this and normal. Plants from the lines giving a low proportion of split cotyledons produced very few affected seedlings from any treatment, and results from these plants have been omitted.

Results

1954 Experiment. The frequency of seedlings with split and whole cotyledons from pollinations made on different dates and plants are given in Table 1.

Table 1

Numbers of Seedlings with Split or Normal Cotyledons from Selfed Swede Plants

Parent		Pollination date											
		29/10		5/11		11/11		19/11		26/11		Total	
No.	Phenotype	N.*	S.†	N.	S.	N.	S.	N.	S.	N.	S.	N.	S.
5241/1	Schizocotylous	6	20	39	33	8	24	15	42	19	29	87	148
2	,,	2	50	27	44	14	54	4	37	8	30	55	215
3	Normal	27	36	32	21	21	3	45	44	26	39	151	143
4	,,	29	40	22	13	43	8	27	29	7	4	128	94
5	,,	24	62	26	9	24	29	13	31	15	11	102	142
6	,,	31	58	34	6	14	13	27	10	10	4	116	91
7	,,	18	7	17	4	73	18	72	13	69	3	249	45
		137	273	197	130	197	149	203	206	154	120	888	878

* N = normal.
† S = seedling with one or two split cotyledons.

There were highly significant differences between pollinations made at different dates, and between plants, the abnormal plants 5241/1 and 5241/2 producing a higher proportion of split cotyledons than the rest, and 5241/7 a significantly lower proportion.

1956 Experiment. The plant which received the high rate of 2·4-dichlorophenoxyacetic acid died without setting any viable seed. Results from the other plants are presented in Table 2.

No convincing differences occurred between the treatments, nor were there any obvious correlations between time of development or position on the plant and the proportion of abnormal seedlings. However, the data permit some conclusions about the processes leading to the production of split cotyledons.

To be scored '3' a cotyledon would have to divide at an early stage of development, a score of '2' would result from a later division and a '1' would result from a division occurring at a late stage of cotyledon development.

Both the cotyledons of one seedling were often affected, and each seedling

TABLE 2

The Degree of Splitting of Both Cotyledons on Seedlings from Different Plants

Parent Plant			Numbers of seedlings with various grades of split cotyledons									
Code number	Treatment		0:0	1:0	1:1	2:0	2:1	2:2	3:0	3:1	3:2	3:3	Total
1020/3	Control		295	61	7	83	22	23	108	16	19	10	644
1029/3	,,		712	151	17	231	39	64	544	59	149	124	2,090
1040/3	,,		401	108	17	210	27	69	470	70	163	78	1,613
1040/1	2·4-D	1 p.p.m.	314	106	22	152	45	71	317	46	100	71	1,244
1020/1	,,	10 ,,	300	126	44	165	65	53	310	64	73	57	1,257
1029/1	I.A.A.	1 ,,	648	80	4	121	23	29	249	21	37	23	1,235
1040/2	,,	10 ,,	568	160	35	113	43	56	392	36	89	49	1,541
1029/2	T.I.B.A.	10 ,,	506	180	50	252	78	92	429	85	141	98	1,911
1020/2	,,	100 ,,	358	119	31	176	62	89	282	57	97	44	1,315
1034/2	,,	1,000 ,,	567	28	1	91	2	8	146	5	7	10	865
Total			4,669	1,119	228	1,594	406	554	3,247	459	875	564	13,715
Expected			4,266	1,361	108	2,221	354	289	3,183	508	829	594	13,713
Deviation			+403	−242	+120	−627	+52	+265	+64	−49	+46	−30	
Mean % deviation from expectation†			1·99	−1·37	0·76	−3·87	0·31	1·80	1·21	−0·40	−0·03	−0·42	
Standard error of deviation			0·34	0·29	0·18	0·53	0·18	0·23	0·44	0·22	0·28	0·24	
t			5·85**	4·72**	4·22**	7·30**	1·72	8·18**	2·75*	1·82	0·11	1·75	

† Calculated from deviations from expectation within each plant progeny.

* Significant—5% level. ** Significant 1% level.

could have two cotyledons in any grade. If each cotyledon of one seedling was affected independently of the other, one would expect a random association of the various grades of abnormality. In Table 2 the numbers of seedlings from each plant with each combination of abnormal cotyledons are listed.

The relative incidence of '0's, '1's, '2's, and '3's from all plants was 0·5577, 0·0890, 0·1452, and 0·2081. From these the expected numbers in each class were calculated to give the total expected in Table 2. The expected number in the '0':'0' class for example was $0{\cdot}5577 \times 0{\cdot}5577 \times 13{,}715 = 4{,}266$, in the '1':'0' class $2 \times 0{\cdot}5577 \times 0{\cdot}0890 \times 13{,}715 = 1{,}361$. These expected values were calculated separately for each plant, and the deviations were analysed, treating the columns as independent. The sum of the deviations from expectation is given in Table 2. There was a significant excess of '0':'0', '1':'1', '2':'2', and '3':'0' combinations, and a deficiency of '1':'0', and '2':'0' combinations. So when both cotyledons were split, they tended to be affected in the same degree.

Discussion and Conclusions

The differences in the proportion of split cotyledons from different plants, and from different lines of swedes, some of which have given less than one in a thousand seedlings with split cotyledons while others have given up to 70 per cent., show that the tendency for swede embryos to produce split cotyledons is inherited, as it is in many other plants (Straub, 1948; de Vries, 1902). The variation in proportions of seedlings with split cotyledons produced at different times on the same plant, which has also been recorded by Straub (1948), Haskell (1954) and Palmer (1957), shows that environmental influences also affect this tendency. The non random association of grades of splitting of both cotyledons on the one seedling shows further that the likelihood of splitting occurring, and the stage of cotyledon development at which it occurs, is a property of the whole embryo, and not of each separate cotyledon.

Cotyledons which split at an early stage of development (scored '3'), were more common than ones that split later, even from plants which produced mostly normal seedlings. Cotyledons were thus more likely to split early in development than later. It is simplest to assume that this splitting results from the same causes which initiate regular cotyledon differentiation, and that in embryos which produce split cotyledons, the conditions conducive to differentiation persist for a longer period than normal in relation to cotyledon development, and a second cycle of differentiation of the cotyledon primordia sometimes occurs.

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Studies in the Physiology of Lichens

I. The Effects of Starvation and of Ammonia Absorption upon the Nitrogen Content of *Peltigera polydactyla*

BY

D. C. SMITH

(*University Department of Botany, Oxford*)

With two Figures in the Text

ABSTRACT

Discs cut from the thallus of the lichen *Peltigera polydactyla* contain relatively large amounts of ammonia nitrogen and small amounts of amide nitrogen. During starvation in the dark the amounts of ammonia and amide nitrogen remain unchanged, but the amount of amino-nitrogen increases. Absorption of ammonia results in increases in the amounts of ammonia and amino-nitrogen in the discs, but not in the amount of amide nitrogen. It is therefore concluded that amides do not have an important quantitative role in the nitrogen metabolism of the discs.

Ammonia absorption is stimulated by addition of glucose to the medium, but the very slow rate of protein synthesis is only slightly increased by glucose. The rate of nitrate absorption is much slower than that of ammonia absorption.

The ecological implications of the results are discussed, and existing knowledge of the nitrogen metabolism of lichens is reviewed.

INTRODUCTION

THE first two of this series of papers describe laboratory experiments on the physiology of discs cut from the thallus of the lichen *Peltigera polydactyla*. The experiments deal mainly with the absorption and subsequent fate within the discs of some simple nitrogenous substances.

Since lichens do not have any special absorbing organs, nutrients are presumably absorbed in solution from the habitat over the whole thallus surface. A knowledge of this process of absorption would thus be of great value in understanding both the physiology and ecology of lichens. Harley and Smith (1956) have emphasized the need for a satisfactory knowledge of the physiology of lichens as functional entities—especially with regard to the understanding of ecological problems. The experiments described by Harley and Smith dealt primarily with sugar absorption by *P. polydactyla*. While sugar absorption has in itself but little ecological significance, the experiments of Harley and Smith were of general importance in several respects. Firstly, they demonstrated that there was little difficulty in the preparation and sampling of lichens for physiological experiments. Secondly, they illustrated the

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potential value of certain techniques such as dissection; and thirdly, they showed that the rates of some metabolic processes—especially sugar absorption—were higher than might be expected from such a relatively slow growing or mature tissue.

The absorption of simple nitrogen compounds is presumably a process that occurs in nature, and hence a knowledge of it may have some immediate ecological significance. Admittedly, there is a strong possibility that *P. polydactyla* can fix atmospheric nitrogen in view of the fact that this property is possessed by the related *P. praetextata* (Scott, 1956) but it is unlikely that this would be its sole nitrogen source in nature. As with other symbiotic structures which can fix nitrogen it can probably utilize such simple nitrogen compounds as it can absorb from the surrounding medium.

A study of the absorption of simple nitrogen compounds has the additional advantage that the immediate fate of these compounds within the tissues can be determined, and thus some guide as to the probable course of nitrogen assimilation can be obtained. This in itself is of value since knowledge of the nitrogen metabolism of lichens is extremely limited, and in fact it can be summarized as follows. (1) There have been a number of estimates of the nitrogen content of lichens which range from 0·75 per cent. of the dry weight for *Usnea barbata* (Sosa-Bourdouil, 1944) to 7·5 per cent. for *Parmelia scruposa* (Weigelt, 1869). However, most values lie within the range 2 to 5 per cent. (Tobler, 1925). Sosa-Bourdouil studied the distribution of nitrogen in the thallus of *U. barbata*, and found that the algal region contained more than the central axis. (2) Nitrogen fixation has been demonstrated in three species of lichens by Bond and Scott (1955) and Scott (1956). It is reasonable to suppose with Scott that at least most of the nitrogen fixation was due to the fact that the symbiontic algae in each of the three lichen species were blue-green. There have been conflicting reports of the presence of *Azotobacter* in lichens (for references on this see Scott, 1956; and Des Abbayes, 1951) but as yet there has not been any demonstration of nitrogen fixation by a lichen with symbiontic green algae from which *Azotobacter* has been isolated. (3) Galinou (1954) examined a wide range of lichens for the presence of the enzymes urease, allantoinase, and allantoicase, and she produced evidence for the existence of one or more of these enzymes in most of the species she studied, concluding that the presence or absence of the enzymes was to some extent correlated with the occurrence of organic nitrogen substances in the natural habitats of these lichens. (4) There have been a few studies of the nutritional requirements of the isolated symbionts in culture (e.g. Quispel, 1945; and Thomas, 1939). These show that on the whole, lichen fungi seem to prefer organic nitrogen sources to inorganic, while lichen algae show similar but perhaps less distinct preferences—possibly because as Quispel has shown, some lichen algae can grow well on inorganic media provided that there are traces of certain vitamins present. (5) Salomon (1914) showed that a number of lichens could absorb nitrate and ammonium ions from culture media; the lichens with green algae took up much more nitrate than those with

blue-green algae although both types appeared more or less equal in their powers of ammonia absorption.

This paper describes the results of analyses for various nitrogen fractions in discs cut from the thallus of *Peltigera polydactyla*, and shows how the distribution of nitrogen between the various fractions is affected by starvation. Also, the absorption of ammonia and its immediate fate within the tissues is described.

Material and Methods

Samples of discs cut from the thallus of *P. polydactyla* were prepared in the manner described by Harley and Smith (1956). For studies of ammonia absorption, samples of 30–50 discs were shaken in the dark in either 20 or 25 ml. aqueous 5 mM solutions of ammonium chloride (analytical reagent grade) in stoppered 100 ml. conical flasks in a water bath at 20° C. for 24 hours. All solutions were buffered to pH 5·6 by M/100 potassium hydrogen phthalate. At the end of the absorption period, the amount of residual ammonia in the medium was estimated by the method described below. Parallel estimates of the total nitrogen in the medium after Kjeldahl digestion were also made, and these always gave the same values as for free ammonia in the medium; it was therefore assumed that no nitrogenous substances were released into the medium by the discs, and this was confirmed by the fact that no nitrogen could be detected in solutions of glucose and buffer in which discs had been shaken for 24 hours.

For estimations of the nitrogen content of the discs, the following extraction procedure was adopted. At the end of the absorption period, samples were washed three times in distilled water, lightly surface dried with filter-paper, and put into 4–5 ml. 80 per cent. alcohol in corked specimen tubes for preservation. Some 5–7 days later the samples were homogenized in N/100 hydrochloric acid at room temperature for 10 minutes. The insoluble matter was then separated by centrifuging and washed twice with N/100 hydrochloric acid. The washings, together with the initial extract and the preserving alcohol, were made up to a known volume, usually 50 ml., and this constituted the extract of 'soluble nitrogen' compounds. The nitrogen content of the residual insoluble matter was then estimated after a Kjeldhal digestion by the methods described below.

The following methods were used to estimate the nitrogenous constituents of both media and extracts of samples:

Ammonia N. Ammonia was estimated by distillation into 1 per cent. boric acid containing an indicator (Conway, 1950) and the boric acid was then titrated against N/100 hydrochloric acid. Distillation was carried out *in vacuo* at 40° C., and ammonia was released from solutions by addition of alkaline potassium metaborate. For some estimates made after Kjeldahl digestion, ammonia was removed from the digests by steam distillation.

Unstable amide N. This was estimated by the increase in free ammonia after hydrolysis at pH 6·5 for 2 hours at 100° C. (Vickery *et al.*, 1935).

Stable amide N. This was estimated by the increase in free ammonia above the level of unstable amide-N after hydrolysis with 2N H_2SO_4 for 3 hours at 100° C.

α-amino N. This was estimated colorimetrically with ninhydrin by the method of Yemm and Cocking (1955) after removal of ammonia from the stable amide hydrolysate. A complete reaction with ninhydrin was assumed, but it must be pointed out that Yemm and Cocking find that some amino-acids such as tryptophane and tyrosine do not give a complete reaction.

Nitrate N and Nitrite N. These were estimated colorimetrically with l-naphthylamine by the method of Nelson, Kurtz, and Bray (1954), nitrate being reduced to nitrite with finely powdered zinc and manganese sulphate. In practice, nitrates and nitrites could not be detected in extracts of lichen discs, although when known amounts of nitrates and nitrites were added to extracts, a 95 per cent. recovery of them was achieved. It was therefore assumed that the combined nitrate and nitrite content of the samples was less than 10 mg. N per 100 mg. dry weight.

Total N. Kjeldahl digestions for the determination of the total soluble and total insoluble nitrogen were carried out by the method of Conway (1950). Ammonia in the digests was estimated by the methods described above.

'Unidentified' soluble nitrogen. In the soluble nitrogen extracts, the difference between the Kjeldahl nitrogen estimate and the sum of values obtained for ammonia, amide, and α-amino N was termed 'unidentified' soluble nitrogen.

The methods of measuring sugar uptake and of respiration rates by Warburg procedures were the same as those described by Harley and Smith (1956).

Experimental Results

Nitrogen Content of Discs

Values for the nitrogen content of discs which were obtained in the experiments are shown in Table 1. The total nitrogen content varied from 3·6 to 4·5 per cent. of the dry weight; the soluble nitrogen component comprised approximately one-quarter of the total nitrogen. The characteristic features of the soluble nitrogen component are that the amounts of ammonia nitrogen are relatively large, and the amounts of amide nitrogen relatively small. Since discs contain approximately 320 mg. water per 100 mg. dry weight when saturated, it can be calculated that the concentration of ammonia in the tissues is of the order of 0·04 Equiv. per l. It is therefore likely that most of the ammonia nitrogen in the tissue exists in the form of weakly ionized salts of organic acids. As pointed out in the previous section, no nitrate nitrogen could be detected in extracts of discs, and it is therefore assumed that the combined nitrate and nitrite nitrogen content of the discs does not exceed 10 mg. N per 100 mg. dry weight.

Effects of Starvation

When discs are starved on water in the dark, there is a gradual and slow rise in the soluble at the expense of the insoluble nitrogen component. This

TABLE I

Nitrogen Contents of Peltigera *Discs. Summary of Analyses of Control Samples Obtained in Experiments*

(All samples were analysed after 24 hours on buffer in the dark. Values for nitrogen in table = μg. N/100 mg. dry wt.)

Experiment Date:	15.5.57	24.6.57	3.9.57	28.10.57	12.11.57	18.11.57	4.2.58
Fractions of soluble Nitrogen							
Ammonia N	223	197	174	167	172	159	188
Unstable amide N	18	28	6	29	44	19	9
Stable amide N	35	15	8				
α-Amino N	273	204	249	322	256	384	383
Unidentified N	425	454	473	336	345	266	500
Total soluble N . .	974	898	910	854	817	844	1,080
Insoluble N . . .	2,748	2,792	2,672	2,940	3,010	2,706	3,446
Total nitrogen . . .	3,722	3,690	3,582	3,794	3,827	3,550	4,526

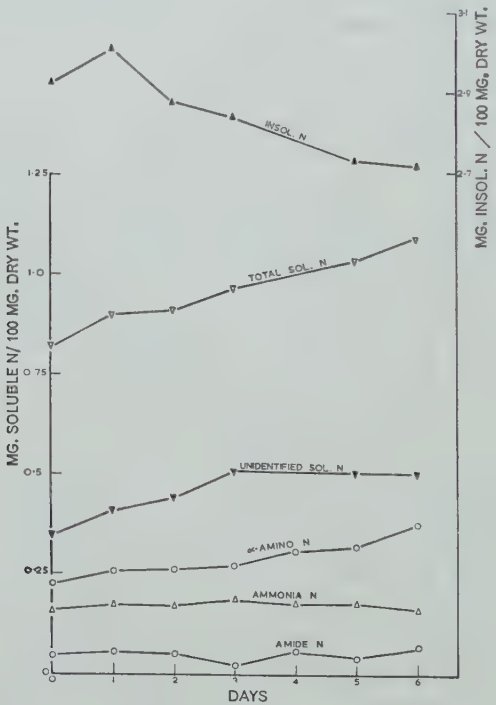

FIG. 1. Changes in the nitrogen content of *Peltigera* discs during starvation on distilled water in the dark at 20° C.

is illustrated in Fig. 1, which is based upon an experiment in which discs were starved over a period of 7 days. The rise in soluble nitrogen is composed mainly of increases in the amino and unidentified nitrogen fractions—the other fractions show virtually no change over the whole period.

It is interesting to compare these results with those obtained when some flowering plant tissues such as barley leaves are starved in the dark (e.g. Yemm, 1937). In the case of barley leaves there is an increase in the amount

of amide nitrogen during the early part of starvation which is followed by an increase in ammonia nitrogen preceding the death of the tissues. Such experiments form part of the evidence that amides are of importance in the metabolism of many flowering plants. Since the amount of amide nitrogen in the lichen discs is initially small, and since it shows no change during starvation, it appears that amides do not have an important quantitative role in the nitrogen metabolism of the discs.

The absorption and fate of ammonia in the discs, and the effects of glucose

Fig. 2 illustrates the progress and amount of ammonia absorption by discs during a 24-hour experimental period, with and without glucose in the medium. It can be seen that in the absence of glucose, there is very little ammonia uptake after 12 hours, but in the presence of glucose, uptake is not only stimulated but also continues at a fairly steady rate throughout the latter part of the absorption period. This result is similar to that obtained by Macmillan (1956) for the fungus *Scopulariopsis brevicaulis*, and by Harley and Budd (unpublished) for *Neocosmospora vasinfecta*.

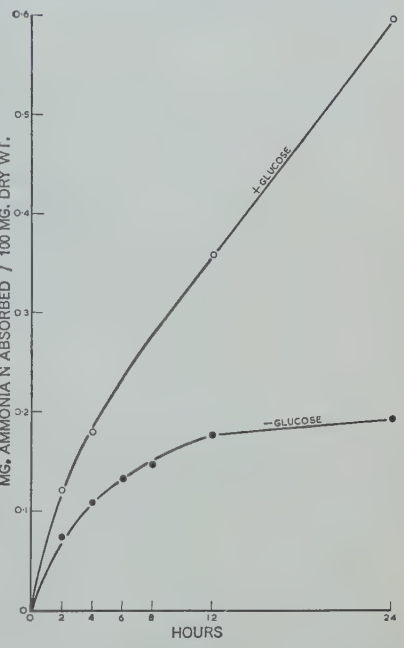

FIG. 2. The progress of ammonia absorption by *Peltigera* discs shaken in ammonium chloride solutions in the presence and absence of glucose. Ammonium chloride = 5 mM; glucose = 14 mM

Table 2 shows the changes in the nitrogen content of discs after 24-hour periods on buffer, glucose, ammonia, or ammonia plus glucose. It can be seen that discs on buffer may show significant increases in the soluble nitrogen component, composed mainly of increases in the amino and unidentified nitrogen fractions; these changes are presumably the effects of starvation. Addition of glucose to the medium reduces the extent of these effects, and also causes a significant reduction in the amount of ammonia nitrogen, indicating that glucose promotes the assimilation of ammonia. There was no significant release of ammonia into the medium by discs on either buffer or glucose.

It can also be seen from Table 2 that absorption of ammonia results in large increases in the ammonia and amino-nitrogen fractions in the discs; the increases are greater in the presence of glucose than in its absence. The amide nitrogen fraction in the discs shows no significant change after ammonia absorption, and this is further evidence that amides do not have an important quantitative role in nitrogen metabolism of the discs. The most likely fate of the ammonia absorbed into the discs is to be combined directly

Table 2

Changes in Nitrogen Fractions of Discs after 24 hours' Incubation on Buffer, Glucose, Ammonium Chloride, or Glucose plus Ammonium Chloride

(NH_4Cl = 5 mM, glucose = 16 mM; all solutions buffered to pH 5·7 by 10 mM phthalate. Temperature = 20° C.

Values for nitrogen in table = μg. N/100 mg. dry wt.)

	Expt. 3.9.57					Expt. 18.11.57					Expt. 4.2.58 (No NH_4Cl in medium)			Difference significant at level $P = 0{\cdot}05$
		− Glucose		+ Glucose			− Glucose		+ Glucose					
	Start of expt.	− NH_4Cl	+ NH_4Cl	− NH_4Cl	+ NH_4Cl	Start of expt.	− NH_4Cl	+ NH_4Cl	− NH_4Cl	+ NH_4Cl	Start of expt.	− Glucose	+ Glucose	
Glucose absorbed from medium (mg./100 mg. dry wt.)	—	—	—	21·9	22·0	—	—	—	25·3	24·2	—	—	39·1	2·4
Ammonia absorbed from medium (μg N/100 mg. dry wt.)	—	—	210	—	584	—	—	188	—	640	—	—	—	54
Fractions of soluble nitrogen: Ammonia N	154	+20	+130	−30	+204	181	−26	+97	−63	+129	235	−47	−101	24
Unstable amide N	10	−3	+2	+3	−9	26	−7	0	−4	+11	0	+9	+12	20
Stable Amide N	10	−2	+2	0	+21									
α-amino N	189	+60	+211	−27	+321	306	+78	+193	+21	+294	356	+27	−74	30
Unidentified N	423	+50	+22	+26	−6	184	+82	+58	+41	+127	281	+219	+151	60
Total soluble N	786	+125	+243	−28	+531	697	+147	+338	+87	+561	871	+209	−11	84
Insoluble N	—	(2672)	(2567)	—	(2879)	2874	−168	−50	−58	+116	3433	−93	+113	186

into α-amino compounds, probably amino-acids. However, a transitory formation of amides during nitrogen assimilation cannot be ruled out on the evidence given here.

The effects of glucose on the uptake and subsequent fate of ammonia in the discs are similar to those observed by Harley and Budd (unpublished) for the fungus *Neocosmospora vasinfecta*, except that these authors find that glucose-feeding results in a marked shift of soluble to insoluble nitrogen, suggesting that glucose promotes protein synthesis. In the case of *Peltigera* discs, feeding glucose causes only a very small shift of soluble to insoluble nitrogen: the increase in soluble nitrogen resulting from ammonia absorption by discs

TABLE 3

Respiratory Response of Discs to Addition of Ammonium Chloride and Glucose

(Rates measured over 2 hours in Warburg apparatus at 20° C., and expressed as μl./hour/100 mg. dry wt. NH_4Cl and glucose both 10 mM.)

Basic rate of oxygen uptake . . .	76±2
Rate after addition of ammonium chloride .	94±3
,, ,, ,, ,, glucose . . .	155±4
,, ,, ,, ,, mixture of glucose and ammonium chloride	161±4

in the presence of glucose is equivalent to 87–90 per cent. of the amount of ammonia nitrogen absorbed from the medium. Thus, factors other than the supply of carbohydrate may be limiting the rate of protein synthesis.

It can be seen from Table 2 that the amount of ammonia nitrogen accumulating in the discs as a result of ammonia absorption is greater in the presence of glucose than in its absence. It may therefore be the case that glucose increases the capacity of the discs to hold ammonia nitrogen; absorption of glucose may give rise to increased rates of glycolysis, so that more organic acids would be produced which would serve to bind ammonia in the tissues.

Data in Table 3 show that absorption of ammonia in the presence and absence of glucose is accompanied by increased rates of oxygen uptake. Yemm and Willis (1954) found increased rates of respiration during nitrogen assimilation in the yeast *Torulopsis utilis*, and concluded that this could be due to three factors: (*a*) the synthetic processes of assimilation may lower the level of high energy phosphate bonds; (*b*), the diversion of electrons for reductive amination reactions may alter the oxidation-reduction conditions within the cells; and (*c*), synthesis of amino acids and proteins may make demands upon the organic acids produced in the course of glycolysis and respiration. It is probable that similar considerations apply in the case of *Peltigera* discs.

Nitrate absorption

Salomon (1914) showed that lichens with blue-green algae absorbed relatively little nitrate compared to those with green algae, although both types

of lichen absorbed similar amounts of ammonia. Preliminary investigations with *Peltigera* discs tend to confirm this in that the amount of nitrate nitrogen absorbed by the discs from 5 mM sodium nitrate solutions was found to be 20 per cent. of the amount of ammonia nitrogen absorbed from 5 mM ammonium chloride solutions. Analysis of discs after a 24-hour period of nitrate absorption showed that 90 per cent. of the nitrate lost from the medium could be recovered as nitrate in the discs.

Nitrogen Fixation

Since Scott (1956) has shown that *Peltigera praetextata* can fix atmospheric nitrogen, it is likely that *P. polydactyla* possesses this property since this lichen also has *Nostoc* as the phycobiont. However, it is unlikely that nitrogen fixation by discs would have any significant effect on the results of the experiments described in this paper for the following reasons. (1) From what is known of the conditions affecting nitrogen fixation by *Nostoc* sp. (e.g. review by Fogg, 1956) the rate of fixation is lower in the dark than in the light, and is suppressed by the addition of available nitrogen compounds to the medium. (2) In none of the experiments described here was any significant increase in nitrogen content recorded over and above any increase due to absorption of ammonia; there was also no significant release of nitrogen into the medium by the discs. The rate of nitrogen fixation recorded by Scott was of the order of 70 μg. N per 100 mg. dry wt./24 hours (including 12 hours' illumination). It is probable that under the conditions of the experiments described here the rate of nitrogen fixation would be much slower than this and would not have a significant effect upon the results.

Discussion

Values for the total nitrogen content of *Peltigera polydactyla* fall into line with the little that is known of the nitrogen contents of lichens, and they are of a similar order to those found by Scott (1956) for the related *P. praetextata*. The distribution of the various nitrogen fractions within the tissues shows no marked differences from what has been found for free-living algae and fungi. There is a relatively large amount of ammonia nitrogen in the tissues, and it is likely that much of it is combined into weakly ionized salts of organic acids.

The amount of amide nitrogen in the discs is very small, and there is no evidence that amides play an important quantitative role in the nitrogen metabolism of the lichen. There is no significant change in the amount of amide nitrogen in the discs either during the breakdown of proteins associated with starvation, or during nitrogen assimilation following upon absorption of ammonia. Although a transitory formation of amides may occur, the probable path of ammonia assimilation is directly into α-amino compounds, probably amino-acids. In this respect the lichen would resemble some free-living fungi in which this problem has been investigated (e.g. Roine, 1947).

There is but little evidence as to the mechanism of ammonia uptake by the discs. The fact that absorption is accompanied by increased rates of oxygen

uptake does not necessarily show that absorption is an active process: ammonia may well enter by free diffusion of the undissociated molecule and the increased respiration rates may in fact result entirely from assimilation of ammonia within the discs. The mechanism of ammonia absorption by free-living fungi has received little attention, but Macmillan (1956) has produced evidence that accumulation of ammonia by the fungus *Scopulariopsis brevicaulis* is a passive process. Macmillan also found that glucose stimulated ammonia absorption by *Scopulariopsis*. A similar effect was observed with *Peltigera* discs, and it may be due to one or more of the following factors: (1) if ammonia absorption by the discs is an active process, then glucose may provide extra energy for it by acting as an additional respiratory substrate; (2) glucose promotes assimilation of ammonia, thus lowering the level of ammonia nitrogen in the tissues and enabling more ammonia to enter the discs—but since more ammonia nitrogen accumulates in the tissues in the presence of glucose than in its absence, then this cannot be the only factor causing the stimulation of ammonia uptake by glucose; (3) glucose increases the capacity of the tissues to hold ammonia nitrogen—it is possible, for example, that ammonia is bound in the tissues by acids produced in the course of glycolysis. With regard to this latter factor, it is interesting to note that Harley and Wilson (*in litt.*) visualize that potassium absorption by beech mycorrhizas is dependent upon the presence and availability of respiratory intermediates which serve to bind potassium in the tissue.

Compared to other types of plant tissues, discs of *P. polydactyla* have relatively very slow rates both of protein synthesis during nitrogen assimilation, and of protein breakdown during starvation. This may well be related to the biology of lichens in nature. Since they are plants which typically inhabit barren habitats, they are probably strongly adapted to withstanding periods of starvation, and also to utilizing absorbed nutrients at a slow rate. This would also suggest that if *P. polydactyla* relies on nitrogen fixation for its main source of nitrogen in nature, it would require only a relatively slow rate of nitrogen fixation.

Summary

1. The nitrogen content of *P. polydactyla* varies from 3·6 to 4·5 per cent. of dry weight. Soluble nitrogen comprises approximately one quarter of the total, and is characterized by relatively large amounts of ammonia and small amounts of amide nitrogen.

2. During starvation in the dark, there is a decrease in insoluble nitrogen and a corresponding increase in soluble nitrogen composed of increases in the amino and 'unidentified' soluble nitrogen fractions. The amounts of ammonia and amide nitrogen show no significant change during starvation.

3. Discs can absorb ammonia from ammonium chloride solutions, and the absorption is stimulated by glucose. Ammonia absorption results in increases in the ammonia and amino-nitrogen fractions in the tissues. There is no significant increase in insoluble nitrogen even in the presence of glucose.

4. It is concluded that amides do not have an important quantitative role in the nitrogen metabolism of the lichens because the amount of amide nitrogen in the tissues is initially small and shows no change during starvation or during ammonia absorption.

5. Nitrate absorption, the mechanism of ammonia absorption, and the relationship of some of the experimental results to the ecology of lichens is also discussed.

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Further Observations on Fungi Inhabiting Pine Stumps

BY

D. S. MEREDITH

(*Botany School, University of Cambridge*)[1]

Abstract

Growth-rates, both on malt agar and in stump wood, of early stump colonizers such as *Fomes annosus*, *Peniophora gigantea*, *Stereum sanguinolentum*, and *Leptographium lundbergii* are higher than those of later-colonizing agarics. The initial invasion of stump roots usually proceeds via the stump body by fungi which infect the cut surface.

Although stumps may dry out appreciably shortly after felling, in later stages of decay the moisture content of the wood often increases considerably. Stump decomposition is associated with a succession of decay fungi, the '*Peniophora*', '*Hypholoma*', and '*Tricholoma*' stages being distinguished. Several phycomycetes and fungi imperfecti colonize decayed, but not fresh, stump wood.

P. gigantea is shown to be a vigorous competitor of *F. annosus* for initial colonization of stumps: it may also replace *F. annosus* in stumps. *Gliocladium viride* and *Trichoderma viride* compete with wood-rotting basidiomycetes and sometimes replace them in very decayed wood.

Introduction

IT has been shown that stumps of plantation pines are infected by several fungi shortly after felling (Meredith, 1959). Frequent early colonists include *Fomes annosus* Fr., *Peniophora gigantea* Fr. (Massee), *Stereum sanguinolentum* Alb. *et* Schw., and *Ceratocystis* spp. Infection by these species occurs by means of air-borne spores impacting on the exposed stump surface, and hence they have been referred to as 'stump-surface colonizers'. Invasion of stump roots by the mycelium of saprophytes present in soil or litter also occurs, but only many months after felling.

The present paper describes studies on the progress of stump colonization, and the succession and interaction of fungi in stumps. The investigation was carried out in East Anglian plantations, where *Pinus sylvestris* L. and *P. nigra* var. *calabrica* Schneid are the species most commonly planted.

Progress of Colonization

Rate of Fungal Spread

The growth-rate of a fungus is an important factor governing the extent to which it colonizes stumps. Growth-rates of several common stump-inhabiting fungi have been determined on malt agar. Thus, Cartwright and Findlay (1934, 1946) found that the optimum temperature for growth of *F. annosus* was about 28° C., the daily increase in diameter of a Petri-dish culture being

[1] Now at Banana Board Research Department, South Ave., Kingston, Jamaica.

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13 mm. Corresponding data for other species were as follows: *P. gigantea*, 16 mm. at 28° C., *Polystictus abietinus* (Dicks.) Fr., 9·8 mm. at 28° C., and *S. sanguinolentum*, 7·9 mm. at 20° C. Lagerberg *et al.* (1927) found that several blue-stain fungi, for example *Ceratocystis* spp. and *Leptographium lundbergii* Lagerberg *et* Melin, had optima between 22° and 25° C. At 25° C. cultures of *L. lundbergii* increased in diameter at the rate of 24 mm. per day.

In the current investigation, growth-rates on malt agar of the above-named fungi were determined. The results are not recorded here since they agree well with those of earlier workers. However, data for three other common stump fungi are given in Table 1. It is seen that these fungi are relatively slow growing with optimal growth temperatures between 22·5° and 25° C.

TABLE 1

Mean Daily Increase in Diameter (mm.) of Colonies of Stump-inhabiting Fungi on Malt Agar

Species	Temperature °C.				
	10	15	22·5	25	28
Hypholoma fasciculare (Huds.) Fr.	3·9	4·2	5·8	5·9	5·1
Paxillus atrotomentosus (Batsch.) Fr.	1·1	2·3	3·3	2·8	2·5
Tricholoma rutilans (Schaeff.) Fr.	3·3	3·7	5·6	5·3	4·8

Since growth-rates on malt agar may bear little relation to those under natural conditions, an experiment was designed to assess the rate of spread of selected fungi in stumps, following infection at the cut surface. Healthy 20-year-old pines were felled in May 1957 and the stumps produced were inoculated immediately with spore suspensions. After 4 weeks some of the stumps were sampled by sawing off a large portion of the stump body: the remaining stumps were sampled after a further 4 weeks. The extent of colonization down the stump was, for each fungus, indicated by a characteristic discoloration of the wood. The results are given in Table 2.

TABLE 2

Growth-Rate of Fungi in Stumps after Inoculation of the Cut Surface with Spores; Mean Values derived from 6 Stumps

Species	Extent of growth (cm.) at intervals after inoculation		Growth-rate (mm. per day)
	4 weeks	8 weeks	
F. annosus	7·6	14·2	2·4
P. gigantea	7·7	13·8	2·2
S. sanguinolentum	6·6	11·3	1·7
L. lundbergii	23·7	$\geqslant$ 30	8·5*

* Growth-rate based on measurements after 4 weeks only.

During the experimental period, air temperatures ranged from a mean maximum of 23·8° C. to a mean minimum of 8·3° C. The relatively greater

extents of growth during the first 4-week period may have resulted from spores being washed some distance into tracheids before germination occurred: this has been demonstrated in the case of *F. annosus* (Rishbeth, 1951). The growth-rate of *F. annosus* agrees well with that observed in infected stump roots (Rishbeth, 1951). The surprisingly high growth-rate of *L. lundbergii* sometimes resulted in lateral roots being colonized after 8 weeks.

Further observations were made to determine the length of time required for fungi to invade stump roots via the stump body. *F. annosus*, *P. gigantea*, *S. sanguinolentum*, and several blue-stain fungi had often grown into stumps for distances of 20–30 cm. from the cut surface after 3 months. In 6-month-old stumps, the same fungi were commonly entering the proximal ends of lateral roots. More distal root regions are not usually invaded by stump-surface colonizers for 12–18 months after felling. In deep sandy Breckland soils, lateral roots are developed at greater depths than in other soil types, with the result that longer intervals are required for their colonization by fungi which infect the cut surface.

It is relevant to note here that other saprophytic stump fungi, for instance *H. fasciculare*, *P. atrotomentosus*, and *T. rutilans*, which commonly invade stump roots via the soil, have relatively low growth-rates in fresh stump wood (Meredith, 1959). This is compatible with the results of growth-rate determinations on malt agar: also it helps to explain why these fungi do not colonize stumps extensively until many months after felling.

Factors Affecting Rate of Stump Colonization

Moisture content of stump wood. The moisture content of wood is of considerable significance in relation to growth of timber decay fungi (Cartwright and Findlay, 1946) and several determinations were carried out on stump wood. Serial sections 2–3 cm. thick were cut from 5-month-old stumps and weighed immediately. The samples were oven-dried at 105° C. until a constant weight was attained; moisture contents of representative stumps, expressed as percentages of oven-dry weights, are given in Table 3.

TABLE 3

Moisture Content of Serial Sections of 5-month-old Stumps; Percentage of Oven-dry Weight

Position of lower surface of section relative to ground level (cm.)	Stump No. 1	2	3	4	Mean %
15	35·5	31·9	—	49·2	38·9
12	49·9	36·8	49·4	82·1	54·5
9	54·4	39·2	50·3	117·8	65·4
6	69·9	44·2	52·8	144·2	77·7
3	89·2	46·7	60·3	155·4	87·9
Ground level	113·6	56·9	69·5	164·5	101·1
−3	—	—	87·4	—	—
−6	141·5	77·6	—	174·6	131·2

These stumps had been exposed to relatively high temperatures and low relative humidities before sampling in the early summer months of 1957. Well-defined moisture gradients were evident, the lowest moisture content occurring at the top of the stump. It is relevant here to note that in some stumps the moisture content was not much above the fibre-saturation point (about 27 per cent.). Cartwright and Findlay (1946) state that the optimum for growth of most wood-rotting fungi lies appreciably above this point. Thus it seems likely that stumps may dry out to such an extent that fungal growth is limited. Some evidence in support of this suggestion is the fact that growth-rates of *F. annosus* and *P. gigantea* in stumps naturally infected at the cut surface during the relatively warm, dry spring of 1957 were significantly lower than those in stumps produced during the cooler and wetter autumn of the same year.

Further moisture content determinations suggested that small stumps dry out more quickly than larger ones during drought. It is possible that the degree of shelter afforded by ground vegetation influences the extent of moisture content fluctuations.

Ring-barking tree prior to felling. Leach (1937, 1939), working in Nyasaland, found that ring-barking forest trees prior to felling had a selective effect on fungal colonization of the remaining stumps. Thus *Armillaria mellea*, which normally colonizes such stumps, was unable to colonize stumps of ringed trees. He concluded that ringing resulted in a depletion of carbohydrates sufficient to limit growth of *A. mellea*, but not that of several saprophytes.

In the present investigation the effect on stump colonization of ring-barking pines was studied. Thirty 18-year-old dominant trees were selected, of which 15 were ring-barked in May 1957, 6 months before felling. Ringing was performed by cutting away a strip of bark 6–8 cm. wide around the circumference of the stem at a distance of about 30 cm. above ground level. The exposed tissues were heavily painted in order to exclude infection. After felling, stumps of ringed and unringed trees were inoculated at the freshly exposed surface with spore suspensions of *F. annosus*, *P. gigantea*, or *L. lundbergii*. Stumps were sampled 14 weeks later and the extent of growth of each fungus was measured. The results obtained showed that for each fungus there was no marked difference between growth-rates in stumps of ringed and those of unringed trees. Ring-barking causes a disturbance in the normal flow of food materials to regions below the ring (Curtis and Clark, 1950). If the concentration of such nutrients influences fungal growth-rates, then it is possible that a longer interval between ringing and felling is required to detect any effects.

Vigour of tree. In another experiment, growth-rates of the three above-named fungi were determined in stumps both of dominant and of very suppressed trees. There was no significant difference between the mean extents of growth in the two stump types, suggesting that the vigour of the tree did not affect the rate of spread of fungi in stumps.

Other factors. Examination of the optimal temperatures for growth on malt agar of several stump-colonizing fungi (p. 64) shows that, under East Anglian conditions, these species are exposed for most of the year to sub-optimal temperatures. It is reasonable to suppose that the rate of stump colonization is determined partly by prevailing temperatures, being greatest under relatively warm conditions. However, it has been suggested that low moisture content of stump wood, especially in warm, dry periods, may limit fungal growth. Interaction of these two factors must be considered when interpreting growth-rate measurements.

Previous observations of Rishbeth (1951), Käärik and Rennerfelt (1957), and Meredith (1959) suggest that high resin content of stump wood increases resistance to infection and subsequent spread in stumps.

Distribution of Fungi in Stumps

Transverse sections from the top of 4-week-old stumps usually revealed the presence of several small, relatively isolated fungal colonies. There is a tendency for such colonies to be elongated in a radial plane. Successive samplings of individual stumps showed that these colonies eventually expand in the horizontal plane with the result that a characteristic sectoring is displayed. Invasion of the stump in the vertical plane proceeds in the form of several spearheads, of which the apices occur at different levels, probably as a result of variations in time of initial infection and in growth-rate. By further growth, fungi enter the tap root and lateral roots. A detailed examination of 48 stumps 12 months after felling showed that *F. annosus*, *P. gigantea*, *S. sanguinolentum*, and various blue-stain fungi were present only in the more proximal root regions. This provided further evidence that these fungi normally colonize stump roots via the stump body and not directly from the soil.

The distribution of fungi in stump roots is related to that in the body of the stump. Serial sections of stumps colonized by several fungi showed that the position of colonies relative to each other underwent little change throughout the length of the stump body. Distribution of fungi over the surface of a transverse stump section thus provides a fair guide to their distribution in lateral roots. In some cases a fungus is excluded from lateral roots by other species which were in a more favourable position to enter them. In the case of *F. annosus*, this will reduce the potential 'infective capacity' (Rishbeth, 1951) of stumps.

The distribution of *Biatorella resinae* (Fr.) Mudd. and *Ophionectria cylindrospora* (Sollman) Berl. *et* Vogl. is of interest. Both fungi form fructifications on the layer of resin which accumulates on the stump surface. Out of a total of 346 6-month-old stumps sampled, *B. resinae* occurred on 279 and *O. cylindrospora* on 232. Fructifications were rarely found on older stumps in which the resin layer had been decomposed.

Other fungi occasionally observed on the surface but rarely in the wood of

young stumps include *Botrytis cinerea* Pers., *Penicillium* spp., and *Trichoderma viride* Pers. *ex* Fr.

Stump Decomposition

Physical Effects

The first superficial indication of stump decay is a loosening of the bark caused by rapid disorganization of the cambium and the phloem- and xylem-initial cells. Loosening proceeds basipetally, and after about 9 months it is often possible to remove easily by hand the complete bark layer.

Although no experiments were carried out to assess the activity of fungi in decomposing stump wood, certain observations may be related to the findings of other investigators. *P. gigantea*, one of the most frequent early stump colonists, can cause considerable loss of weight in wood maintained under laboratory conditions (Cartwright and Findlay, 1946: Käärik and Rennerfelt, 1957). Wood from 2-year-old stumps colonized extensively by *P. gigantea* is very decayed and there is little doubt that the fungus plays an important part in early stages of decomposition. By contrast wood from stumps of similar age, but colonized by *F. annosus*, is much sounder. This observation is compatible with the finding of Cartwright and Findlay (1946) that *F. annosus* caused only small weight losses in pine blocks. The results of studies on sap-stain (Cartwright and Findlay, 1946) suggest that many of the blue-stain fungi which colonize stumps play only a minor part in decomposition.

In East Anglian plantations, where stumps belong chiefly to trees 18–30 years old, decay is usually complete after 8 or 9 years from the time of felling. It is common to find stump remains in the form of a hollow shell of bark, woody tissues having been reduced to a humus-like material. Exceptions to this are very resinous stumps, which are decayed very slowly.

The moisture content of stumps of different ages was determined in the manner described previously: the data are shown in Table 4. Moisture content tended to increase with age of the stump. The wood of stumps more than about 5 years old is often waterlogged; it is possible that the oxygen tension is reduced to such an extent that fungal growth is limited.

Table 4

Moisture Content (expressed as percentage of oven-dry weight) of Samples of Stump Wood; Means Derived from 6 Samples.

Age of Stump (years)	Moisture content of wood		
	Min.	Mean	Max.
1	48·7	69·5	97·0
2	111·7	120·9	130·0
3	121·6	164·5	246·7
4	57·8	123·2	169·8
5	148·0	228·7	393·5

Associated Fungi

Stumps of various ages were sampled in order to determine the frequency with which different species of decay fungi occurred. Since it was difficult to identify certain species by mycelial characters alone, the presence of their sporophores was used for recording purposes. The data obtained from a total of 873 stumps sampled are shown in Table 5. The results suggest that there is a sequence of decay fungi in stumps shortly after felling, the flora at first being dominated by stump-surface colonizers such as *P. gigantea*, *F. annosus*, and *S. sanguinolentum*. Later, several agarics appear and, in turn, become the dominant species. Thus, *H. fasciculare* was most frequent in 3- and 4-year-old stumps and *T. rutilans* in those more than 5 years old. Further observations suggest that succession in small stumps is more rapid than that in large ones. For example, after 4 years *P. gigantea* was the dominant species in stumps of 26-year-old trees having stem diameters of 15–25 cm., whereas in smaller stumps *H. fasciculare* or *T. rutilans* were dominant.

TABLE 5

Frequency of Basidiomycetes in Stumps of Different Ages; Percentage of Stumps Containing Fungus

Fungus	Age of stumps (years)					
	1	2	3	4	5	8
**Fomes annosus*	43	37	16	7	17	14
**Peniophora gigantea*	85	89	24	3	—	—
**Stereum sanguinolentum*	32	29	26	15	6	5
**Polystictus abietinus*	—	4	35	13	33	—
**Hypholoma fasciculare*	—	—	45	55	24	12
Tricholoma rutilans	—	—	15	43	73	66
Polyporus amorphus	—	—	5	8	4	—
Paxillus atrotomentosus	—	—	—	—	5	4
P. involutus	—	—	—	—	—	1
Collybia maculata	—	—	—	—	4	1
Clitocybe aurantiaca	—	—	—	—	—	3
Flammula sapinea	—	—	—	3	5	2
Pholiota flammans	—	—	—	—	—	1

* Fungi identified by mycelial characters: presence of remaining fungi indicated by sporophores.

The first sporophores to appear on stumps are generally those of *P. gigantea*; these were observed 7 months after felling. Sporophores of *F. annosus* and *S. sanguinolentum* develop after 12–18 months. In stumps which had been sampled by cutting off a section from the top, it was a common occurrence for sporophores of the above-mentioned fungi to develop after a shorter interval. A similar precocious development of sporophores was induced by removing the bark from stumps immediately after felling. These observations suggest that the bark and the wood at the stump surface form a temporary barrier to sporophore formation. Fruiting bodies of several agarics appear on older stumps, especially during late summer and autumn. Since

they are susceptible to freezing they perish after the first autumn or winter frosts.

Considerable difficulty was sometimes experienced in isolating decay fungi from old stump wood because of rapid overgrowth of the agar surface by moulds. The abundance of these moulds in stump wood was determined by aseptically transferring small wood fragments to the surface of acidified malt agar, and incubating for 5–7 days at 22·5° C. The information obtained from 350 stumps is recorded in Table 6. Phycomycetes and fungi imperfecti were very infrequent in stumps less than 1 year old: however, their frequency increased markedly after 2 years. The commonest species were *Mucor ramannianus* Möll., *Mucor* spp., *Penicillium* spp., *Gliocladium viride* Matruchot, other *Gliocladium* spp., and *T. viride*. It is probable that ascomycetes and basidiomycetes were more frequent in old stump wood than was indicated by this technique. The use of selective agar media (Russell, 1956) may bear this out.

TABLE 6

Frequency of Different Classes of Fungi Isolated from Fragments of Stump Wood Removed at Intervals after Felling; Percentage of Samples from which Fungi Developed

	Age of stumps					
	6 months	1 yr.	2 yrs.	3 yrs.	4 yrs.	5 yrs.
Basidiomycetes	76	79	15	20	16	15
Ascomycetes	14	13	0	0	0	0
Phycomycetes	0	8	13	32	26	5
Fungi imperfecti	4	10	80	60	64	88
Sterile samples	14	6	0	4	0	0

INTERACTION OF STUMP-INHABITING FUNGI

Interaction of F. annosus *and* Peniophora gigantea

A study of the interaction of these fungi was prompted by the finding of Rishbeth (1952) that *P. gigantea*, a purely saprophytic species, exercised a degree of biological control over stump infection by *F. annosus*.

The effect of inoculating stumps with various proportions of spore inoculum of *F. annosus* and *P. gigantea* was investigated. Spore suspensions in sterile water were prepared from freshly collected sporophores and brought to a concentration of *c.* 1×10^5 spores per ml. by means of a haemocytometer. Suspensions of each fungus were mixed in various ratios and thoroughly shaken. Stumps of healthy 20-year-old Scots pine were inoculated immediately after felling by spraying 5 ml. of mixed suspension from an atomizer on to the surface. Inoculum controls were provided by stumps inoculated with *F. annosus* or *P. gigantea* only, while stump controls were inoculated with sterile water. Stumps were then covered with sheets of moisture-proof 'Cellophane', secured by rubber bands. Stump covers were removed after 4

weeks in order to prevent over-growth by *T. viride*. After a further 8 weeks, wood sections were cut off the stumps, incubated at 22·5° C. in moist containers, and the extent of colonization by *F. annosus* and *P. gigantea* assessed after 6–8 days. The results are given in Table 7. *P. gigantea* had a decisive advantage in competition for colonization of stumps inoculated with approximately equal proportions of spore inoculum. Germination tests showed that the percentage viability of *F. annosus* spores was considerably lower than that of *P. gigantea* spores. However, the difference was not sufficiently large to invalidate the conclusion that *F. annosus* is unlikely to become dominant in stumps also infected by *P. gigantea* unless the balance of infecting basidiospores is greatly in its favour. Results obtained by Rishbeth (1952) suggest that different conditions may exist if oidia of *P. gigantea* are the infective propagules.

TABLE 7

Colonization by F. annosus *and* P. gigantea *of Stumps inoculated with Mixed Spore Suspensions*

Proportions of spores *F. annosus*:*P. gigantea*	Abundance* *F. annosus*	After 12 weeks *P. gigantea*
F. annosus only	80	2
100:1	23	60
10:1	7	87
5:1	3	90
2:1	7	90
1:1	2	95
1:2	2	90
1:5	0	100
1:10	< 1	98
1:100	0	95
P. gigantea only	0	92
Control, uninoculated	0	4

* Abundance was the mean percentage area colonized in 5 wood sections.

In a further experiment the ability of *F. annosus* to colonize stumps already infected with *P. gigantea* was determined. Healthy Scots pine were felled in October 1957 and 30 stumps were inoculated by spraying with spores of *P. gigantea*. Of these, 5 were further inoculated immediately with *F. annosus* spores. Stumps were then covered in the manner previously described. Further lots of 5 stumps were inoculated with *F. annosus* at intervals. The concentration of the *F. annosus* spore suspensions was approximately one hundred times greater than that of the *P. gigantea* suspension. This measure was designed to increase the chance of obtaining infection by *F. annosus* of stumps also inoculated with *P. gigantea* (Table 7). Stump and inoculum controls were set up as in the previous experiment. Stumps were sampled 6 weeks after the final inoculation and the extext of colonization by each fungus was determined after incubating samples in the manner described by Rishbeth (1951). The results are shown in Table 8. The ability of *F. annosus* to colonize stumps decreased rapidly as the interval between the respective

inoculations increased, and the fungus was apparently unable to compete successfully with *P. gigantea* when the latter had been established for more than 1–2 weeks.

TABLE 8

Infection by F. annosus *of Stumps Previously Inoculated with* P. gigantea; *Abundance of Fungi 6 weeks after Inoculation with* F. annosus

	Stumps inoculated with							
	F. annosus at intervals (weeks) after inoculation with *P. gigantea*					*P. gigantea* only	*F. annosus* only	Not inoculated
	0	1	2	3	5			
F. annosus	37	5	< 1	0	0	0	83	< 1
P. gigantea	59	87	96	100	100	92	14	16

The converse of the previous experiment was set up to determine whether *P. gigantea* is able to colonize stumps already occupied by *F. annosus*. Experimental details were similar to those previously described except that the order of inoculation was reversed: also, basidiospore suspensions were brought to approximately the same concentration. The results are shown in Table 9. *F. annosus* became dominant when inoculation with *P. gigantea*

TABLE 9

Infection by P. gigantea *of Stumps Previously Inoculated with* F. annosus; *Abundance of Fungi 6 weeks after Inoculation with* P. gigantea

	Stumps inoculated with							
	P. gigantea at intervals (weeks) after inoculation with *F. annosus*					*P. gigantea* only	*F. annosus* only	Not inoculated
	0	1	2	5	7			
F. annosus .	4	52	71	88	72	0	73	0
P. gigantea	88	47	26	10	16	97	15	21

was delayed for longer than a week. The occurrence of *P. gigantea* in stumps inoculated with this fungus 2 weeks after felling may have been a result of natural infection shortly after felling, since stump controls were infected to a similar extent. It was concluded that *F. annosus* has a decisive advantage in competition for colonization if it becomes established more than a week before infection by *P. gigantea* occurs. The relative frequency of the two species in later stages of colonization is affected by their competition, as discussed below.

Rishbeth (1950, 1951) has shown that *P. gigantea* replaces *F. annosus* in lengths of pine root maintained under laboratory conditions, and also in stumps. In the current investigation replacement in plantation stumps was investigated in more detail. In two previously unthinned plantations healthy 20-year-old Scots pines were felled. The stumps produced were sampled after 3–5 months by cutting wood sections 2–3 cm. thick from the top. The same

stumps were re-sampled after a further 10–16 months, and in addition root samples were taken from some. The data obtained from the samplings are recorded in Table 10. In Table 10 (*a*), it is seen that the frequency of *F. annosus* decreased markedly during the interval between successive samplings; in contrast that of *P. gigantea* increased slightly. Since other fungi were very uncommon in these stumps, it is suggested that *P. gigantea* had partly replaced *F. annosus*. The results shown in Table 10 (*b*) indicate that replacement had proceeded downwards in the stump.

TABLE 10

(*a*) *Percentage of Stumps sampled* (*out of 280*) *Containing* F. annosus *and* P. gigantea *in the Body of the Stump*

Interval between felling and sampling (months)	*P. gigantea* (total)	*F. annosus* (total)	*P. gigantea* and *F. annosus*	*F. annosus* only
3–5	78	41	35	6
13–21	84	15	10	5

(*b*) *Percentage of Stumps* (*out of 62*) *Containing* F. annosus *and* P. gigantea *in Different Parts of the Stump*

Interval between felling and sampling (months)	Sample type	Fungus	
		P. gigantea	*F. annosus*
3–5	Stump body	77	43
13–21	Stump body	80	23
13–21	Roots	80	40

Further evidence of such replacement was obtained from stumps 8–13 months old. Stump sections were incubated at 22·5° C. and examined at intervals to map the distribution of *F. annosus* and *P. gigantea* over the cut surface. A characteristic feature was the presence of dark zone-lines, which formed boundaries where the two species met. In sections which were kept for several weeks it was observed that *P. gigantea* grew through wood colonized by *F. annosus*, but not vice versa. It would appear that zone-lines do not form an impregnable barrier to competitors of *F. annosus*. A similar conclusion was drawn by Rishbeth (1951), working on the interaction of *F. annosus* and *T. viride* in stump roots: he found that zone-lines checked, but did not prevent, replacement of *F. annosus* by the latter species.

Interaction of F. annosus *and* P. gigantea *with Other Stump Fungi*

Evidence that *P. gigantea* replaces other fungi was obtained from sections of stumps 1–2 years old. Blue-stained wood was frequently colonized by this fungus and attempts to isolate blue-stain fungi were usually unsuccessful. In other stumps there was extensive development of zone-lines between colonies of *P. gigantea* and *S. sanguinolentum*. By maintaining stump sections under laboratory conditions it was possible to observe the replacement of *S. sanguinolentum* by *P. gigantea*.

Although *F. annosus* often colonizes blue-stained wood, there was no evidence that any replacement occurs: several blue-stain fungi, including *Ceratocystis* spp., could usually be isolated from such wood. On the other hand, *F. annosus* is unable to colonize wood infected with *S. sanguinolentum* and it was observed that these fungi may exist together in stumps for considerable periods, especially when *P. gigantea* was absent.

Replacement of *F. annosus* by *T. viride* has been described by Rishbeth (1950, 1951). Similar observations were made during the current investigation, especially in stumps more than 2 years old. In addition, *T. viride* occasionally replaces *P. gigantea*, *P. abietinus*, and *S. sanguinolentum*; in these instances there was a development of zone-lines similar to that described above. Replacement by *T. viride* normally proceeds from the stump roots towards the body of the stump and only rarely in the opposite direction.

G. viride may colonize stumps which are also infected with decay fungi or blue-stain species. Although the fungus was occasionally isolated from 6-month-old stump wood, it was most common in stumps more than 2 years old. Failure to isolate wood-rotting basidiomycetes from such partially decayed stumps, or to induce their mycelia to grow out of the tissues by incubating wood in moist containers, suggested that *G. viride* had replaced them. This possibility was tested by inoculating sections of healthy pine stem with mixed spore suspensions of *G. viride* and *F. annosus* or *P. gigantea*. Sections were incubated at 22·5° C. in Petri-dishes and small wood fragments were removed at intervals and transferred to an agar surface. It was found that *F. annosus* or *P. gigantea* colonized the wood initially, whereas *G. viride* developed only in the cambium and phloem. However, after 3 weeks' incubation, *G. viride* had overgrown the sections entirely and it was impossible to isolate the basidiomycetes from the wood. It was concluded that *G. viride* cannot compete successfully with *F. annosus* or *P. gigantea* for initial colonization of a fresh woody substrate, but that it may do so when decay has reached a certain stage.

Colonization of Stumps of Dead Trees

Certain fungi may be already established in stumps of dead trees at the time of felling (Meredith, 1959). The ability of other fungi to colonize such stumps was investigated.

At monthly intervals after felling sections were cut from the top of stumps belonging to trees which had been killed by suppression. Many of these stumps were already colonized at the time of felling by *P. abietinus*, *S. sanguinolentum*, or blue-stain fungi. It was found that only rarely did these stumps become infected by *F. annosus* or *P. gigantea*. An experiment was set up, therefore, to test the ability of these fungi to infect such stumps after inoculation at the cut surface. Dead suppressed trees bearing sporophores of *P. abietinus* or *S. sanguinolentum* were felled, and the stumps produced were inoculated immediately with a spore suspension of the test fungus. Stumps of other dead trees, colonized instead by blue-stain fungi, were

similarly inoculated. Inoculum controls were provided by stumps of trees which were suppressed but still living and not invaded by saprophytes. Samples were taken after 6 weeks to determine whether infection by the inoculants had occurred. The results are given in Table 11.

TABLE 11

Ability of F. annosus *and* P. gigantea *to Colonize Stumps of Dead Suppressed Trees Containing Other Fungi at the Time of Felling; No. of Stumps (out of 10) Infected after Inoculation*

Stumps inoculated with:	Stumps of dead trees colonized by *P. abietinus*	Stumps of dead trees colonized by *S. sanguinolentum*	Stumps of dead trees colonized by *Blue-stain*	Control (stumps of healthy trees)
F. annosus . . .	0	0	2	7
P. gigantea . . .	1	0	1	9

Infection by *F. annosus* and *P. gigantea* of stumps of dead trees was associated with the presence of small areas of apparently healthy wood. There was no indication that they had invaded tissues already colonized by other fungi.

In another experiment trees which had been killed by *F. annosus*, as indicated by the presence of sporophores, were felled. Each stump was then inoculated by spraying the cut surface with a spore suspension of a selected fungus. The same suspensions were used to inoculate controls, that is, stumps of healthy trees. After 3 months the number of previously infected stumps (out of 10) colonized by the selected species were as follows: *L. lundbergii* 4, *P. gigantea* 4, and *S. sanguinolentum* 2. The corresponding numbers for healthy stumps were 10, 9, and 9 respectively.

The presence of *F. annosus* in stumps at the time of felling appeared to limit the infective capacity of the saprophytes tested. In the instances where inoculation was successful, the saprophytes were localized in tissues which were free from *F. annosus* at the time of inoculation. There was no evidence that these fungi had infected tissues already occupied by *F. annosus*. These observations suggest that *F. annosus* has a marked competitive advantage in stumps of trees which it has killed. It is possible that the fungus survives in such stumps for longer periods than in those which it infects after felling.

DISCUSSION

During the course of stump decay certain successional changes occur in the fungal flora. In this study three well-defined stages in succession were recognized. The '*Peniophora*' stage was characterized by an abundance of stump-surface colonizers such as *P. gigantea*, *F. annosus*, *S. sanguinolentum*, and various blue-stain fungi, including *Ceratocystis* spp. These fungi normally remain dominant for 2–3 years after felling. In the '*Hypholoma*' stage several agarics appear, of which *H. fasciculare* is often the most common. In addition, polypores such as *P. amorphus* and *P. abietinus* may develop.

Stump-surface colonizers show a decline in frequency. The '*Tricholoma*' stage represents the final period of stump decomposition. Many agarics may be observed, but *T. rutilans* is usually the dominant species. Occasionally, *F. annosus* persists to this stage. The investigations of Käärik and Rennerfelt (1957) suggested that successional changes occur in spruce and pine stumps in Sweden. Thus, *P. gigantea* was most frequent in stumps less than 3 years old, whereas in older stumps several other species dominated the fungal flora. From an examination of the data obtained by these workers it is evident that successional changes extended over longer periods than those observed in the current investigation. This may be related to the fact that they studied stumps of much larger trees.

In the current investigation it was found that the number of phycomycetes and fungi imperfecti increased as stump decomposition progressed: the commonest species observed were *Mucor* spp., *Gliocladium* spp., and *T. viride*. The presence of these fungi sometimes prevented the isolation of basidiomycetes from old stump wood, and a similar difficulty was experienced by Käärik and Rennerfelt (1957). It is interesting to compare the capacity of different fungi to decompose cellulose and lignin, which together constitute a large proportion of the wood substance. Phycomycetes are generally unable to decompose either of these fractions and are restricted to relatively simple carbon compounds such as sugars (Siu, 1951). Many fungi imperfecti, including *Trichoderma* spp., can decompose cellulose but not lignin (Siu, 1951), while most stump-colonizing basidiomycetes can utilize both substrates (Cartwright and Findlay, 1946). The chemical effects of decay in wood were investigated by Lutz (1930) who concluded that the process is similar to hydrolysis in that various intermediate breakdown products such as simple sugars are formed. At certain stages of decay, the rate at which hexoses and pentoses are formed may exceed the rate of utilization by the decay fungus. It is possible, therefore, that the development of non-cellulolytic fungi in decayed stumps is related to a similar accumulation of simpler carbon compounds.

Comparison of the fungal succession in stems of fallen deciduous trees (Mangenot, 1952) and in pine stumps shows that for both the primary colonists are chiefly lignin-decomposing fungi. Development of 'sugar fungi' (Burges, 1939) in late stages of decomposition also occurs in both. These processes may be contrasted with the sequence of fungi in dead plant tissues in the soil. Garrett (1951) has surveyed the relationship of certain soil fungi to their substrates. The pioneer colonists of dead, moribund, or injured plant material are the 'saprophytic sugar fungi', the lignin-decomposing fungi being the last to develop.

Observations and experiments have shown that *P. gigantea* is a vigorous competitor of *F. annosus* for colonization of freshly cut stumps. In stumps inoculated with equal proportions of spores of each fungus *P. gigantea* had a decisive advantage, which was decreased only when the balance of spores was greatly in favour of *F. annosus*. Seasonal variation in the relative proportions

of spores present in the air influences the incidence of stump infection by *F. annosus* (Meredith, 1959). During autumn and winter months, *P. gigantea* exercises some degree of biological control of stump infection by *F. annosus*. Further control is exercised during later stages of colonization, since *P. gigantea* frequently replaces *F. annosus* in stump wood. Rishbeth (1952) found that stumps inoculated with approximately equal numbers of spores of *F. annosus* and *P. gigantea* were usually dominated by the latter 12 months after inoculation.

Experimental inoculations have shown that certain fungi are more successful in competition for colonization if they infect stumps before other species. Similarly, Brooks and Moore (1926) found that plum branches which had been cut back and inoculated immediately with spores of *Stereum purpureum* were rapidly invaded by the fungus. However, when inoculation was delayed for several weeks, branch stumps were colonized by other micro-organisms and *S. purpureum* was excluded. These workers suggested that other micro-organisms had altered the nutritive conditions of the wood and had therefore derived a competitive advantage. Further investigation is desirable to elucidate the nature of competition between stump-colonizing fungi.

Summary

Growth-rates on malt agar of several stump-inhabiting fungi are compared: values for *Fomes annosus*, *Peniophora gigantea*, and *Stereum sanguinolentum* are higher than those for several agarics. There is no marked difference between the growth-rates in stump wood of *F. annosus* and *P. gigantea*; *Leptographium lundbergii* is shown to spread rapidly in stumps. The above-named fungi often invade the proximal ends of lateral roots within 6–9 months of infection at the stump surface. There is no evidence that ring-barking trees 6 months before felling influences the rate of spread in stumps of *F. annosus*, *P. gigantea*, or *L. lundbergii*.

Stumps may dry out appreciably during warm, dry conditions. Moisture gradients were observed in 5-month-old stumps, the lowest moisture content occurring in wood at the top of the stump.

Stump decomposition is often complete 8–9 years after felling. The moisture content of stump wood tends to increase as decay progresses. During the course of decay, a succession of fungi develops. Three fairly well-defined stages in succession, the '*Peniophora*', '*Hypholoma*', and '*Tricholoma*' stages, were distinguished. The numbers of phycomycetes and fungi imperfecti, for instance *Mucor* spp., *Gliocladium* spp., and *Trichoderma viride*, increase with age of stump.

P. gigantea is a vigorous competitor of *F. annosus* for colonization of freshly cut stumps, and became dominant in stumps inoculated with approximately equal numbers of spores of each species. However, the ability of *P. gigantea* to colonize stumps is markedly reduced if *F. annosus* is already established at the time of infection. *P. gigantea* may replace *F. annosus* in stumps, replacement commonly proceeding in the direction of the stump roots.

Gliocladium viride and *T. viride* occasionally replace wood-rotting basidiomycetes in stumps.

Stumps in which other fungi have been established for some time are unlikely to be colonized extensively by *F. annosus* and *P. gigantea*. Also, stumps of trees killed by *F. annosus* are colonized by saprophytes to a lesser extent than stumps of healthy trees.

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Occurrence of Endosperm Haustorium in *Cannabis sativa* L.

BY

MANASI RAM

(*Department of Botany, University of Delhi, India*)

With thirteen Figures in the Text

ABSTRACT

The development and structure of the chalazal endosperm haustorium in *Cannabis sativa* are described. The endosperm is nuclear and a haustorium is formed at the chalazal end. The latter remains free nuclear throughout. Enucleate vesicles appear in the upper part of the endosperm but finally they merge with the cytoplasm of the haustorium. As the embryo reaches maturity it occupies the whole seed cavity, the haustorium collapses and the endosperm persists only as a thin layer.

THE earlier studies on the embryology of *Cannabis* have already been summarized by Schnarf (1931) and, as far as I am aware, there has been no recent work. The ovary bears a single, apically attached, anatropous, bitegmic, and crassinucellate ovule. The development of the embryo sac conforms to the Polygonum type and the endosperm is nuclear (see Schnarf, 1931).

While studying the effect of maleic hydrazide on *Cannabis sativa*, I observed the formation of a chalazal endosperm haustorium both in treated and untreated plants. The development of the haustorium is described here.

The mature embryo sac occupies almost the entire length of the ovule. Repeated divisions of the primary endosperm nucleus result in a large number of peripherally distributed nuclei (Figs. 1–3). The divisions are more frequent in the central part and the embryo sac expands laterally so that it becomes distinguishable as a bulbous middle region, a narrow micropylar, and a tubular chalazal portion (Figs. 4, 5, 7–9). The entire endosperm increases in bulk keeping pace with the enlarging ovule. The tubular extension becomes richly cytoplasmic and functions as a haustorium (Figs. 5, 7–9, 11). Due to rapid nuclear divisions in the upper and middle parts of the endosperm (endosperm proper), the size of the nuclei there remains smaller compared with those of the haustorium which are twice or thrice as large in diameter (Fig. 11). The diameters of the nuclei in the endosperm proper and haustorium are compared in the following table:

	Average diameter of nuclei in microns	
Stage of embryo	Endosperm proper	Haustorium
Heart-shaped . .	8	16
Torpedo . . .	8	23
Nearly mature . .	7	25

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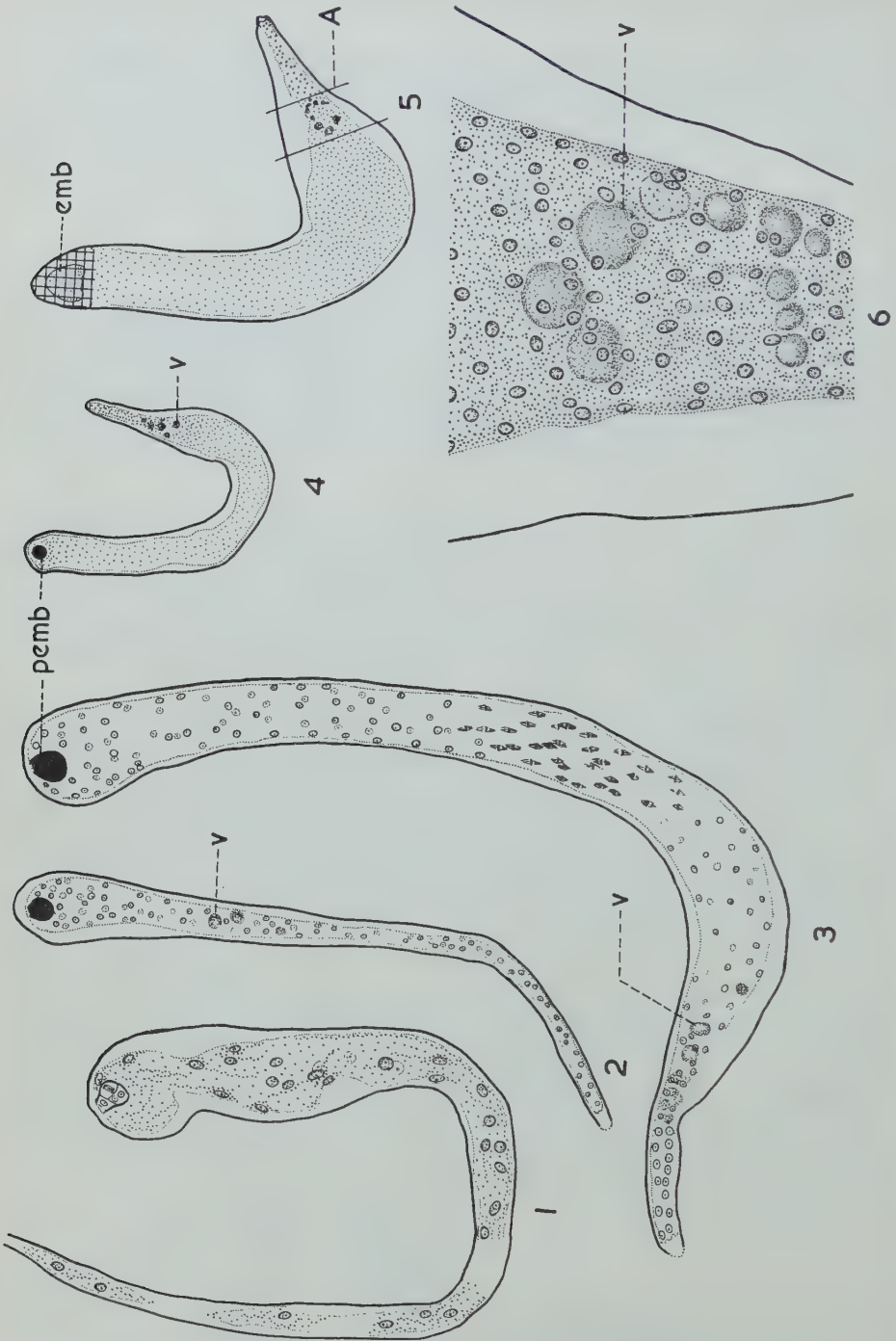
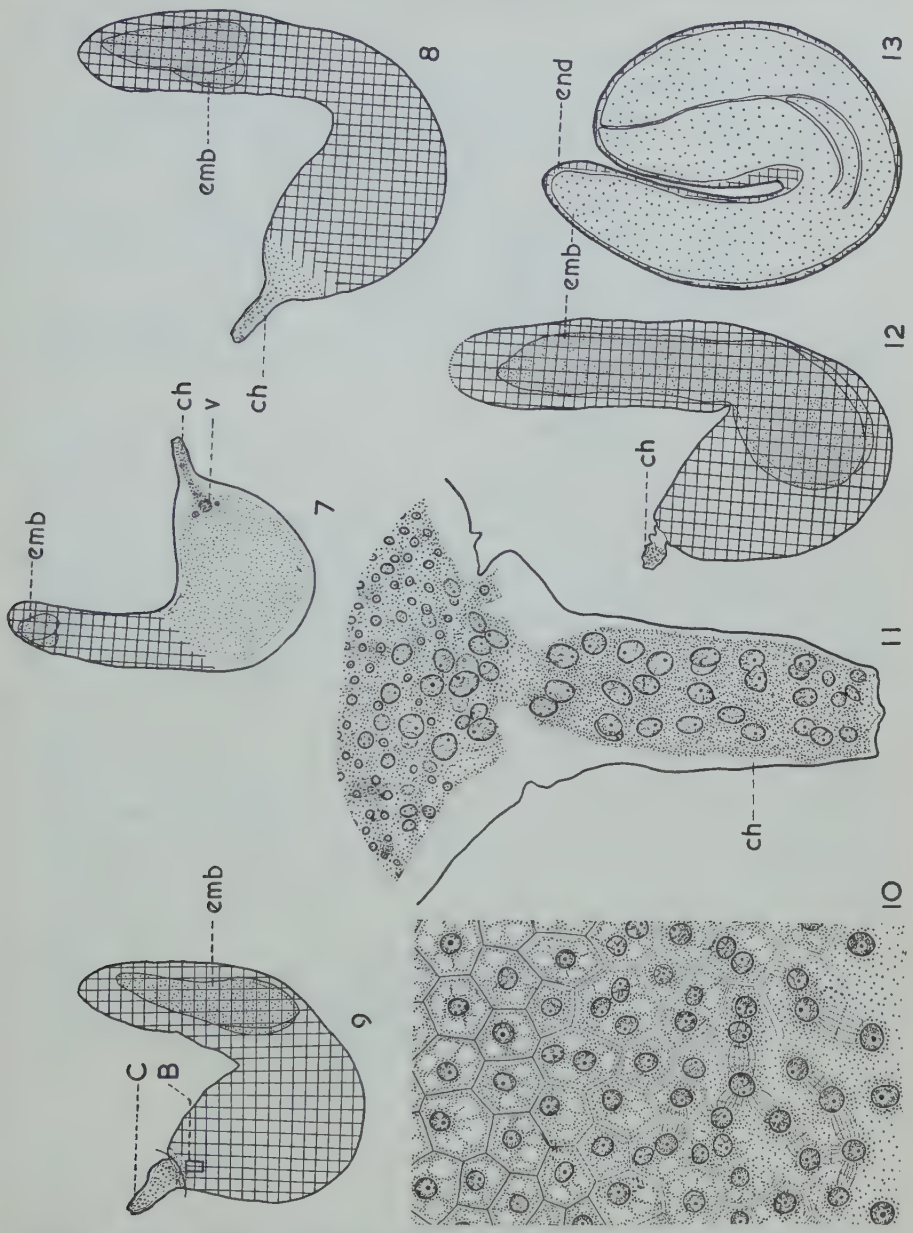


FIGS. 1–13. All figures from dissected whole mounts. *ch*, chalazal haustorium; *emb*, embryo; *end*, endosperm; *pemb*, proembryo; *v*, vesicle. Fig. 1. Embryo sac with 4-celled proembryo and free nuclear



endosperm (×382). Figs. 2–4. Endosperms at early and late globular stages of the proembryo; note the endosperm vesicles. Figs. 2, 3 (×180) and Fig. 4 (×56). Figs. 5, 7–9. Progressive stages of cell formation in the endosperm proper (×56). Fig. 6. Endosperm vesicles enlarged from portion marked A in Fig. 5 (×382). Fig. 10. Magnified view of wall formation in the endosperm from region marked B in Fig. 9 (×700). Fig. 11. Enlargement of haustorium marked C in Fig. 9 (×382). Fig. 12. Endosperm with degenerated haustorium (×56). Fig. 13. Same with mature embryo, the remnants of the haustorium are no more distinguishable (×56).

Another interesting feature is the formation of enucleate vesicles in the upper part of the endosperm (Figs. 2–7) which later migrate downwards and merge with the cytoplasm of the haustorium.

The endosperm remains in a nuclear state up to the globular stage of the proembryo (Figs. 1–4). Thereafter, wall formation is initiated in the vicinity of the embryo which progresses downwards (Figs. 5, 7–9) until the entire endosperm, except the haustorium, becomes cellular (Fig. 12).

In the basal region of the endosperm proper the cells are richly cytoplasmic and contain two to four nuclei (Figs. 9, 10). In the micropylar region the cells are comparatively smaller and vacuolate.

With the maturation of the embryo the haustorium collapses and the endosperm proper is consumed except for a thin papery layer which persists in the seed (Fig. 13).

A perusal of embryological literature on the order Urticales indicates that a chalazal endosperm haustorium has so far been reported only in *Urtica pilulifera* and *U. cannabina* (Urticaceae). There is no record of its occurrence in the Moraceae, Ulmaceae, or Cannabinaceae (see Schnarf, 1931).

On the basis of short and straight stamens; apical, anatropous ovule; and absence of latex, Hutchinson (1926) and Rendle (1952) removed the genus *Cannabis* from the family Moraceae and assigned it to a new family Cannabinaceae. The occurrence of endosperm haustorium in *C. sativa* is an additional distinguishing character between these two families.

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Physiological Investigations on the Banana Plant

I. Biochemical Constituents Detected in the Banana Plant

BY

F. C. STEWARD, A. C. HULME,[1] S. R. FREIBERG,[2]
M. P. HEGARTY,[3] J. K. POLLARD, R. RABSON,[4]
AND R. A. BARR

(*Department of Botany, Cornell University, Ithaca, New York*)

With nine Figures in the Text

Abstract

The interest and economic importance of a better understanding of the physiology and biochemistry of the banana plant is emphasized. Methods are described which are to be applied to the detection and determination of the nitrogenous constituents of alcohol-soluble extracts, and for the detection and analysis of keto acids and non-volatile organic acids. The classes of nitrogen compounds to which attention is given are the amino-acids and their amides, certain volatile amines, ureido and guanido compounds, and certain indole compounds. In view of the large amount present in fruit of the variety Gros Michel from Honduras, free histidine was isolated and critically identified. The use of various reagents and tests for these various classes of substances on paper chromatograms is described. The range of substances which have been detected by these means in the banana leaf, leaf-sheath, fruit (pulp and peel), and stem exudate are indicated by their location on paper chromatograms. Evidence is presented which indicates qualitatively the degree of prominence of the different substances in the tissues or extracts which have been examined. When substances were recognized but not yet identified, they are designated by suitable codes, the conditions of their occurrence are noted, and their distinguishing characteristics are given. This descriptive evidence on the biochemistry of the banana plant provides the background for the more quantitative work which is to follow.

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[1] Present address, Ditton Laboratory, East Malling, Kent, England.
[2] Present address, United Fruit Co., Norwood, Mass., U.S.A.
[3] Present address, C.S.I.R.O. Laboratory, Brisbane, Australia.
[4] Present address, Dept. of Biology, University of Houston, Texas, U.S.A.

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1. General Introduction

THE purpose of this series of papers, of which this is the first, is to present a comprehensive study of the physiology and biochemistry of a particular plant, in this case *Musa acuminata* cv. *Gros Michel*, the principal cultivated banana of the western hemisphere. A few comprehensive studies of other economically important plants have been made, e.g. investigations on tobacco, published under the series title Chemical Investigations of the Tobacco Plant [for earlier references see Part IX (Vickery and Meiss, 1953)]; work on the nitrogen nutrition of *Narcissus* (Vickery *et al.*, 1946) and work on the rhubarb plant (Vickery *et al.*, 1939). However, such investigations are comparatively few, since the tendency has been to select a particular plant for the investigation of a selected physiological process or function. Extensive cultivation of the banana plant under conditions of specialized agricultural practice has produced an increasing number of nutritional,

developmental, and pathological problems. A more comprehensive knowledge of the physiology and biochemistry of this plant should help to interpret these various aspects of its behaviour.

Physiological investigations on the banana were made as early as the beginning of the nineteenth century (Fourcroy and Vauquelin, 1807), only one year after Vauquelin first reported asparagine in asparagus juice. Investigations since that date have been almost entirely restricted to various aspects of the parthenocarpically developed fruit and the conspicuous changes to be observed in the fruit on ripening, such as the conversion of starch to sugar (Buignet, 1861; Ricciardi, 1892; Gore, 1915; Poland *et al.*, 1938; Wardlaw *et al.*, 1939*a*; Barnell, 1940; Lulla and Johar, 1955); the disappearance of tannin (Buignet, 1861; Barnell and Barnell, 1945); the sequence of dramatic respiratory changes associated with the climacteric (Gerber, 1896; Gore, 1915; Wardlaw and Leonard, 1940; Leonard, 1941); and, finally, the conspicuous role played by such hydrocarbons as ethylene, first as products of the metabolism and secondly as active agents which modify the respiration of the fruit (Regeimbel *et al.*, 1927; Hartshorn, 1931; Wolfe, 1931; Gane, 1937; Nelson, 1939). Other biochemical constituents of the fruit which have been investigated are cellulose and hemicellulose (Barnell, 1943*a*); pectin, protopectin, and protein (Stratton and von Loesecke, 1930); pigments (von Loesecke, 1929; Simmonds, 1954); vitamins (Thornton, 1943); various enzymes (Mierau, 1893, Vines, 1903; Bailey, 1912; Onslow, 1920; Falk and McGuire, 1922; Nelson and Hemperly, 1933; Sastri and Row, 1934; Tager and Biale, 1958); and certain organic acids (Harris and Poland, 1937; Lulla and Johar, 1954; Wolf, 1958). To this substantial body of information may be added papers emanating mainly from the School of Tropical Agriculture at Trinidad (Wardlaw *et al.*, 1939*a*; Barnell, 1941, 1941*a*, 1943; Leonard and Wardlaw, 1941; Leonard, 1947); and the Research Laboratories of the United Fruit Company (Niederl *et al.*, 1938; Harris and Poland, 1939; Freiberg, 1955; Rowan, 1959; Henderson *et al.*, 1959). Notwithstanding all this, there is a notable lack of information on parts of the plant body other than the fruit, and on aspects of metabolism other than those which concern, or are immediately related to, carbohydrate metabolism and respiration. Exceptions to this rather broad generalization are the works of Belval (1932) and of Koursanov and Manskaja (1935) on the carbohydrate changes that occur in the banana leaf; and of Cain (1956) and Freiberg and Payne (1957) on the foliar absorption of urea; and of Freiberg and Payne (1957) on the urease activity in banana plants. Aside from rather general observations on the changes in broad categories of nitrogen compounds, there is very little information on the nitrogen metabolism of the banana plant as a whole. The development of newer methods, such as paper chromatography, radioautography, and spectrofluorimetry, now makes it more feasible than hitherto to investigate the constituents of the various parts of the plant body as these are affected by nutrition, development, and environmental factors.

This group of papers will be mainly concerned with a parthenocarpic

cultivar of banana (Gros Michel), although seeded and dwarfed cultivars and species also occur, and some other species of *Musa* are also important, or interesting, in particular ways. *M. paradisiaca*, the plantain, is important as a staple food, and *M. textilis*, the cultivated abaca, is important as the source of Manila hemp. A number of related genera are important as ornamentals, notably *Heliconia*, *Ravenella* (traveller's palm), *Strelitzia* and, though more remotely related, *Canna* of more temperate climates is familiar. Any approach to the biochemistry and physiology of the more economically important banana plant, therefore, attracts attention also to these other plants.

The special features of the morphology and development of the Gros Michel cultivar of *M. acuminata* are as follows. First, this species forms a massive underground storage organ, or rhizome, which functions as an organ of perennation by forming lateral buds or 'tillers' by which the plant is commonly propagated vegetatively. The longevity of the plant, under favourable circumstances, is potentially unlimited, and there are on record examples of continuous growth in cultivation for over 30 years. The first shoots to develop from the newly planted rhizome form smaller plants than the subsequent ones. A special feature of the growth of these buds is the rapid elongation of the *pseudostem*, which is strictly the rolled, overlapping leaf bases of the shoot. As Skutch (1927) stressed, a single leaf of the banana plant is one of the largest photosynthetic units in the plant kingdom. Also, although these leaves are originally entire and simple in shape, they exhibit a marked polymorphism—shown by the changing size and shape of successive leaves. Leaves which spring from a rhizome at the base of a pseudostem are long, pointed, and more lanceolate, but, if these side shoots are excised and propagated free from the rhizome, they will bear leaves which rapidly become more rounded, and, as the shoot grows upward and leaves successively emerge, their size increases quite markedly. This sequence clearly presents problems of morphology which require interpretation in terms of biochemistry and nutrition.

Even more important morphological features are associated with the events leading up to fruit formation. The vegetative-shoot apex, deeply imbedded at the base of the pseudostem, undergoes changes that initiate a floral shoot. In the cultivar Gros Michel the vegetative-shoot apex grows upward between the rolled leaf bases of the pseudostem, and by the time the apex of the true stem has reached an elevation of approximately 1 foot, floral initiation occurs. Very rapidly all the floral parts are then formed, and by the time the inflorescence emerges from the pseudostem, the floral axis has elongated very greatly.

The changes which occur successively in this growing point are of great physiological interest: these concern the transition from a purely vegetative growing point to one which produces, in succession, three different types of flowers: first, those flowers which produce the parthenocarpically developed fruit; next, hermaphroditic flowers, which in the usual case are shed (occasionally they may remain attached to the inflorescence); and, finally, distinctively male flowers, which normally absciss. Nevertheless, the terminal growing-point

of the shoot continues to grow and to form bracts, with male flowers in their axils, although these also absciss. This growth can continue for a very long time, even until the growing tip reaches the ground from a point often 10 or 15 feet above. In normal cultural practice the tip of the inflorescence is removed to increase somewhat the weight of the fruit that develops. This sequence of morphological events presents interesting physiological problems in the biochemical and hormonal determination of the events which have been described and the way they may be affected by environmental and nutritional factors.

The first paper of this group presents a qualitative survey of the biochemical constituents of the plant, particularly those that can be recognized by the techniques of chromatography and spectrofluorimetry. The results reported accrue from the examination of the parthenocarpic fruits ('pulp' and 'peel') at various stages of development, ripening, and senescence; of leaves under various conditions of nutrition and development; of roots and rhizome; and of tissue from the rolled leaf bases of the pseudostem. The methods used permitted the detection of a variety of nitrogen compounds that react on paper with ninhydrin (i.e. mainly amino-acids, amides, and certain volatile amines, and some still unidentified compounds) present in the alcohol-soluble fraction and in hydrolysates of the protein. Using Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), urea and certain ureides can be detected, and also certain indole-like and polyphenolic compounds. By modification of the ninhydrin reaction, guanido compounds can also be detected. Of the non-nitrogenous compounds that are detectable on paper chromatograms, the principal attention has been directed to the organic and keto acids, the latter being detected after conversion to their hydrazones and after catalytic hydrogenolysis to amino-acids. The more precise methods by which this survey was conducted will now be described.

2. Methods

Preparation of Banana Extracts for Chromatography

A. *Non-volatile alcohol-soluble nitrogen compounds*

Fresh material, irrespective of its nature, was weighed and extracted in the cold with 70 per cent. alcohol. If the material was obtained in the tropics, it was placed in alcohol of the required density before shipment. Repeated extraction in the cold removed virtually all of the alcohol-soluble materials without decomposing any heat-labile substances. To insure complete extraction of sparingly soluble substances, a further hot alcohol extraction was made. The combined alcohol extracts were then evaporated in the cold to dryness, small volumes of extracts were concentrated in an air stream at room temperature, while large volumes were concentrated in a flash evaporator at an external bath temperature not exceeding 40° C. The actual temperature of evaporation was of course lower than this, thus preventing the break-down of heat-labile substances. The concentrated residue from alcohol extraction, containing sugars, organic acids, amino-acids, salts, and other minor

constituents was then treated as indicated below, while the alcohol-insoluble residue was retained for hydrolysis of the protein.

The dried alcoholic extracts were partitioned between water and chloroform to remove pigments and fatty material. The chloroform extracts were discarded. The aqueous solutions were reconcentrated to dryness and redissolved in a very small volume of water to which was added a few drops of chloroform as an antiseptic. Extracts were stored in small stoppered vials in the cold until required. If the extracts did not contain excessive amounts of sugars or organic acids, they were suitable for direct chromatography on paper, as will be described. However, in some cases, notably extracts of the ripe fruit, this was not feasible, and interfering substances were removed by the use of resins as follows.

A small column (that is, a few centimetres long) of Dowex-50 resin of 4 per cent. cross-linkage was prepared in the acidic form, using 2N HCl, and it was then washed with water until free from excess of acid. In deciding on the size of the column, allowance must be made for cations (e.g. potassium) other than those of the amino compounds present. To facilitate the even distribution of the amino-acids on the column, it was found effective to add 1 ml. of 2N ammonium hydroxide which neutralized the upper quarter of the column. As the amino-acids move through this neutral zone, they distribute evenly on the acidic layers below. A concentrated plant extract, normally containing approximately 2 to 3 milligrams of nitrogenous substance was then applied to the column. By thorough washing of the column all neutral substances and anions, including sugars and organic acids, were removed; only the cationic materials adhered to the column. The amino acids on the Dowex-50 column were then removed by passing 50 ml. of 2N ammonium hydroxide through the column, and evaporating this effluent to dryness to remove ammonia; the residue was redissolved in a known volume of water. Since 2N NH_4OH does not elute inorganic cations from the column, these also are removed during the treatment. This material was retained for chromatographic examination of the nitrogen compounds by the methods that are indicated below.

B. *Volatile amines*

An aliquot of the original alcohol extract of the tissue was diluted with water, made alkaline with 20 per cent. NaOH to which NaCl was also added. This mixture was steam distilled and the volatile amines collected in 2N HCl. The distillate was evaporated to dryness a number of times to remove hydrochloric acid and then dissolved in a known volume of water.

C. *Non-volatile organic acids*

After the alcohol extracts were passed through a Dowex-50 resin as described at A above, the effluent contained the organic acids and the sugars. This acidic solution was then passed through a 'Deacidite G' column in the OH form, which is a weakly basic anion exchange resin, to absorb all the organic

acids. The organic acids were fractionally displaced from the column with 0·1N HCl, this procedure being continued until chloride passed through the column. The eluate was then evaporated to dryness (first under reduced pressure at 40° C. and then in a stream of air at room temperature), and if it contained appreciable amounts of HCl, was redissolved in water and re-evaporated to dryness to remove the free HCl. Volatile organic acids, if any remained, would be lost at this point. The dried samples were then redissolved in a small known volume of water.

D. *Keto acids*

This important class of metabolites can be detected on paper chromatograms after conversion to amino-acids. For this purpose the plant material was treated as follows.

First, the plant material was treated with an acidic, alcoholic solution of 2, 4-dinitrophenylhydrazine after the manner described by Towers, Thompson, and Steward (1954). This treatment stabilizes the acidic carbonyl compounds as their hydrazones which may then be removed by extraction with aqueous sodium carbonate and then transferred into organic solvent after acidification. The mixed hydrazones in the concentrated organic solvent are then converted into their corresponding amino-acids by catalytic hydrogenolysis. The virtue of this procedure is twofold. First, it stabilizes these otherwise unstable compounds in the form of their hydrazones, and, secondly, the ultimate identifications can rest upon a large body of existing information that relates to the behaviour of ninhydrin reactive substances on paper chromatograms.

E. *The exudate from cut banana shoots*

The liquid which exudes copiously from cut pseudostems was collected at the time of fruit harvest from plantations in Honduras. Toluene was added after collection as a preservative. This exudate turns very dark soon after collection. In this condition the material was forwarded to Cornell University.

A sample of this material was reduced virtually to dryness in a flash evaporator at 40° C. and drying was completed in a desiccator; 25·8 g. of dry matter being obtained from 840 ml. of exudate.

Part of this dry material was extracted twice with 70 per cent. alcohol in a Waring blendor at room temperature and then once in hot 70 per cent. alcohol. These alcoholic extracts were pooled, reduced in volume at 40° C., and to a concentration of 6·1 g. per 100 ml.

F. *Protein hydrolysates*

Banana tissue previously extracted with alcohol as above, was now hydrolyzed with 6N HCl in an autoclave at 15 pounds pressure. It is recognized that tryptophan is lost by this procedure. Any humin formed was removed by centrifugation and the acid extract was evaporated to dryness on a sand bath. After repeated addition of water and redrying to remove excess acid, the

amino-acid residues were again dissolved in water and prepared for chromatography in a manner similar to that used for the alcohol-soluble substances.

Chromatographic procedures

A. *Solvents*

The paper partition chromatography procedures were those in use in this laboratory (Dent *et al.*, 1947; Thompson *et al.*, 1951; Steward *et al.*, 1955). Briefly the procedures were as follows:

For purely qualitative purposes the Whatman #1 ($18-\frac{1}{4}'' \times 22-\frac{1}{2}''$) chromatographic paper was used. A known amount of extract (approximately 50 μg. of nitrogen) was applied to the paper. The first solvent used was phenol/water at pH 5·5. After drying, the second solvent was either collidine-lutidine-water (3 : 1 : 1) or *n*-butanol-acetic acid-water (9 : 1 : 2·9). The former solvent combination has particular advantages for the amides and basic amino-acids. Furthermore, if sugars are present, these can be segregated from the amino-acids on the chromatogram when collidine-lutidine is the second solvent. (This is particularly important in subsequent experiments which involved C^{14}, since confusion of C^{14}-labelled sugars with amino-acids can be avoided.) The conditions of chromatography were controlled to reproduce accurately the positions of known substances on the chromatograms, so that by reference to such internal standards as alanine, precise mapping of constituents could be done.

The extracts containing the organic acids were submitted to one-directional descending chromatography using one of three different solvent systems: each of which moved a distance of about 45 cm.

(1) Butanol-formic acid-water in the ratios 4 : 1 : 5.
(2) Normal propanol-ammonia-water in the proportions 6 : 3 : 1.
(3) Benzyl alcohol-tertiary butanol-isopropanol-formic acid-water in the proportions 3 : 1 : 1 : 1 : 1 (Stark, Goodban, and Owens, 1951).

Malic and citric acids were used as reference standards and aconitic, tartaric, isocitric, succinic, tricarballylic, malonic, glutaric, α-keto-glutaric, glucuronic, phosphoric, quinic, oxalic, glycollic, and glyoxylic acids were used for appropriate comparisons.

B. *The reagents used and the types of compounds detected on chromatograms*

(i) *Ninhydrin.* For qualitative purposes the air-dried chromatogram was covered evenly with a 1 per cent. alcoholic solution of ninhydrin applied from the fine tip of a wash bottle; it was then heated in an air oven at 60° C. Under these conditions aspartic acid and β-alanine appear bright blue, and other compounds have characteristic colours. The chromatograms were then examined qualitatively, and the various coloured reaction products related by position to alanine. Any unidentified ninhydrin-reactive compounds were noted, designated by letter and number, and their position on the chromatogram noted. After the use of ninhydrin, the identity of certain substances

(e.g. pipecolic acid) on the chromatograms may be confirmed by using an ultraviolet lamp. The volatile amines and keto acids as their corresponding amino-acids were also determined by their reaction with ninhydrin, appearing as violet colours on the chromatograms, after the same type of chromatography as that described above.

(ii) *The extended ninhydrin procedure.* This procedure depends on the use of alkali after the chromatogram has been treated with ninhydrin as described above. The paper, after having been treated with fresh ninhydrin solution, is treated evenly with a solution of 0·5 per cent. NaOH in 90 per cent. methanol and redried. Certain additional substances now react with ninhydrin. This is particularly true of guanido compounds, which give a blue-violet colour as follows: Guanidine itself reacts immediately after the NaOH treatment, mono-substituted guanidines like γ-guanidobutyric acid react as soon as the paper is dry, while disubstituted guanidines like creatine and trisubstituted guanidines like creatinine react weakly after prolonged heating at 90–100° C. The latter treatment also causes sugars on the chromatograms to react. The particular merit of the extended ninhydrin procedure over other reagents for detecting guanidines lies in the fact that it enables additional information to be obtained from the chromatograms after use for amino-acid identification.

(iii) *Ehrlich's reagent* (p-*dimethylaminobenzaldehyde*). The Ehrlich's reagent (2 per cent. *p*-dimethylaminobenzaldehyde in 1N alcoholic HCl) was applied in a manner similar to chromatograms prepared in the manner indicated above. After the treated chromatogram is air-dry, certain substances may appear on the paper as variously coloured spots. Generally ureides give a yellow colour, and indole compounds react pink. Certain phenolic substances, e.g. leucoanthocyanins, also react with the reagent, as do aromatic amines. In this laboratory the chromatographic positions of a number of known substances have been established relative to urea (Freiberg *et al.*, 1957), and this information forms the background for the interpretation of the chromatograms obtained from extracts of natural substances.

(iv) *Brom Cresol Green.* The thoroughly dried chromatograms of organic acids were first scanned with an ultraviolet lamp to reveal any fluorescent organic acids, probably phenolic, which had not been removed by the earlier charcoal treatment. Next they were sprayed with a solution of Brom Cresol Green at pH 7. Acidic substances produce yellowish spots against a purple background. Chromatographic identification of a given acid is regarded as definite only if the acid in question matches the position of the authentic substance in all three solvent combinations.

(v) *Ultraviolet fluorescence*: Fluorescent substances may be detected after they are eluted from specified regions on the chromatograms using the recently developed Aminco-Bowman Spectrophotofluorometer. By means of this instrument the wavelength which activates fluorescence may be detected and, at that wavelength, the emission spectrum of the fluorescent light can be automatically traced, either visibly on a Cathode-Ray Oscillograph or graphically using a recorder. Even more sensitive characterization is possible

by repeating this procedure at different pH's. The types of compounds for which this procedure is particularly applicable are generally cyclic, unsaturated substances, particularly those with hydroxyl substitution in the ring.

C. *Chromatographic maps*

After the information on the chromatograms has been assembled by the various methods described above, it is convenient to document and represent it in the form of maps of the kind that have previously been published (Steward, Zacharius, and Pollard, 1955), and which are used as illustrations in this paper.

3. Compounds Detected in the Banana Plant

Non-Volatile Alcohol Soluble Nitrogen Compounds[1]

The various parts of the banana plant contain a large number of free amino-acids and amides which vary considerably as to their relative proportions (Table 1). Furthermore, the total amount of free amino-acids and amides detected may vary from only 20 μg. in the root to about 1,000 μg. per gram fresh weight in the leaf. Of these identified amino-acids, glutamic acid, α-alanine, γ-aminobutyric acid, aspartic acid, glutamine, asparagine, and arginine predominate in one or more organs of the plant. Leaves are conspicuous by their comparative lack of glutamine, which is abundant elsewhere, and by the predominance of glutamic acid, alanine, and γ-aminobutyric acid. Fruit pulp, on the other hand, is conspicuous by the presence of amides and of histidine. Pipecolic acid occurs in most of the organs, as does tyrosine; the former accumulates in certain parts of the plant as illustrated in Table 1, yet is absent from the normal leaf. It is recognized, however, that the failure to report the presence of a given amino-acid may be due, not to its absence, but its being present in an amount too low to detect even with the sensitive methods used in this investigation. Data to be published in a later paper show, however, that pipecolic acid will reach predominating amounts in leaves grown under certain conditions of nutritional imbalance, particularly magnesium deficiency. Histidine is a special feature of the fruit and it may accumulate there in rather large amounts.

Summarizing, the various parts of the banana contain nearly all the amino-acids which one would expect to find free in plants and a number of unidentified amino-acids which will be discussed shortly in section 'D'. The one unique feature of the amino-acid picture is the high concentration of histidine in the fruit pulp. The changes which histidine undergoes on ripening will be discussed in a later publication.

[1] This section is based largely on the work of Freiberg and Barr, with some reference to data obtained by Hulme on the fruit pulp.

Table 1

Identified Ninhydrin Reactive Substances which Occur Free in Banana Plants (Gros Michel)

Amino-acid[1]	Leaf[2]	Leaf sheath[3]	Developing floral parts: ovary	Developing floral parts: style	Fruit[4]: Peel	Fruit[4]: Pulp	Rhizome: Cortex	Rhizome: Stele	Roots[5]: Cortex	Roots[5]: Stele	Roots[5]: Entire
Aspartic acid	+	++++	+	+	++	++	+	+	+	++++	++
Glutamic acid	++++	++++	+	+	+	+	+	+	+	++	++++
Serine	++	++	+	++	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	++	+
Asparagine	++	+	++++	++++	+++	++++	+	—	—	—	—
Threonine	+	+	+	+	+	+	+	+	+	+	+
Alanine	++++	++	++	+	+++	+	+	+	++	+	++
Glutamine	+	++	+++	+++	++	+++	++++	++++	++++	++++	+
Histidine	—	—	+	—	—	+++	—	—	—	—	—
Lysine	+	—	+	+	+	+	—	—	—	—	—
Arginine	+	++++	++++	++++	+	+	—	—	—	—	++++
Methionine sulfoxide	—	—	+	—	+	—	—	—	—	—	—
Proline	++	—	+	—	+	+	+	+	+	+	—
Valine	+	+	+	+	+	+	+	+	+++	+	++
Leucines	+	—	+	+	+	+	—	+	+	+	+
Phenylalanine	+	—	+	+	+	—	—	—	—	—	—
Tyrosine	+	—	+	+	—	+	+	+	+	+	—
γ-Aminobutyric acid	++++	—	+	+	++++	++	++	++	+	++++	++
Pipecolic acid	—	+	++	++	++	++	+	+	+	+	—
Total amino-acids μg./g. fresh weight	1,086	83	811	589	265	515	201	202	77	272	20

In addition to the above, the volatile amines ethanolamine and putrescine have been detected in the banana leaf—the latter only in the potassium deficient leaf.

[1] When the specified amino-acid represents less than 5% of total amino-acids = +; 5–10% = ++; 10–15% = +++; greater than 15% = ++++.
[2] Second youngest fully expanded banana leaf of plant growing in sand culture in Honduras.
[3] The rolled basal portion of the leaf which extends for the length of the pseudostem.
[4] Fruit harvested in the tropics at time for shipment.
[5] Cortex and stele obtained from sturdy mature roots of established plants grown under field conditions in the tropics; entire, young, actively growing roots obtained from banana plants grown in sand culture at Cornell University.

The volatile amines[1]

While the volatile amines have long been conspicuous in bacterial metabolism, they have received less attention in higher plants. More recently, however, attention was directed to these substances by the work of Richards and Coleman (1952) and Coleman and Hegarty (1957) on the presence of putrescine in barley, particularly under conditions of potassium deficiency.

Extracts made from normal banana leaves contain a volatile amine which was identified as ethanolamine on the basis of co-chromatography with the authentic substance. Ethanolamine occurs on the chromatograms at R_f values in phenol and collidine-lutidine of 0·67 and 0·36 respectively. Potassium-deficient banana-leaf tissue yields, in addition to ethanolamine, two other volatile amines. One of these has been identified as putrescine on the basis of its co-chromatography with authentic material.[2]

Isolation and identification of histidine[3]

In the first chromatograms made of the ripening pulp of the mature banana fruit, a strong ninhydrin reactive spot appeared on the chromatograms in the general area of the basic amino-acids. This substance occurred in such quantity that it was at first regarded as an unidentified substance. In view of the amount of this substance in the fruit, a complete isolation, purification, and identification was undertaken, although the substance was quickly recognized chromatographically as the basic amino-acid histidine. Because this amino-acid is usually not found in quantity in the free state and because the examples of its isolation and critical identification are few this work was completed as follows.

An alcoholic extract, representing something over 2 kilograms of fruit, was used for this isolation. The concentrated solution was first extracted with chloroform to remove fatty material, and then shaken with charcoal deactivated with acetic acid after the procedure of Schramm and Primosigh (1943). This procedure removes phenolic substances which may interfere with the subsequent separation of amino-acids on ion exchange columns (Hulme, 1953). The clear pale-yellow liquid was filtered and passed down a 3-tier Dowex-50 ion-exchange column following the general procedure of Partridge and Brimley (1952). The amino-acids were displaced from the column with 0·15N caustic soda and separated into a number of fractions using a fraction collector. After one-directional chromatography, the fractions which contained the 'unknown' were detected and combined. This yielded a concentrated extract containing the bulk of the substance in question, along with some γ-aminobutyric acid and smaller amounts of other basic amino-acids. Further purification was then carried out as follows.

The fraction containing the substance suspected to be histidine was

[1] This section is based on work done by Hegarty and Freiberg.

[2] Dihydroxyphenylethylamine, the decarboxylation product of DOPA, is now identified by Griffiths (*Nature*, 1959; 184, 58) as the substrate of banana oxidase.

[3] This section is based on work done by Hulme and Pollard.

absorbed on a small column containing 4 g. of Dowex-50, 4 per cent. cross-linkage, 200 to 400 mesh, and then displaced from the column by 0·15N ammonia. By this procedure the γ-aminobutyric acid was eluted first, followed by the suspected histidine. More basic amino-acids remained on the column. When the ammoniacal aqueous liquid containing the substance desired was concentrated, the crude substance crystallized. After recrystallization from aqueous alcohol, 25 milligrams of chromatographically pure material were

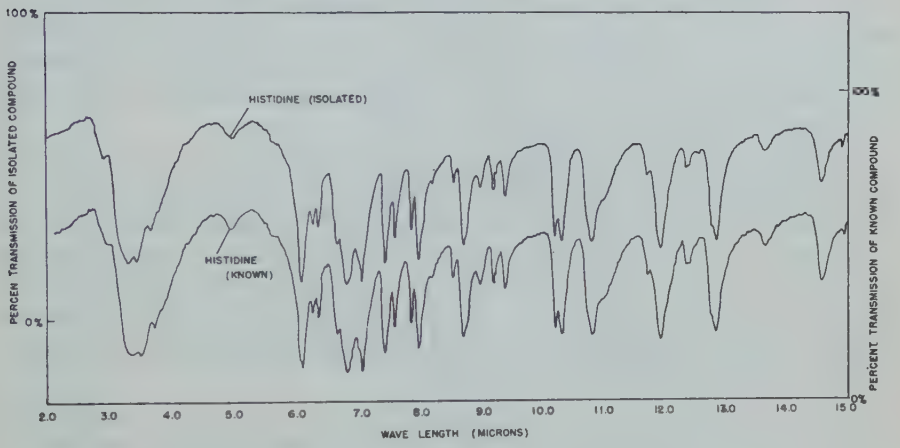


Fig. 1. Histidine infra-red absorption spectrum.

obtained. No attempt was made to isolate exhaustively all of this material from the tissue. This substance is identified as histidine from the following evidence:

1. Authentic histidine and the substance isolated from the banana were indistinguishable in two-dimensional chromatograms using phenol:collidine-lutidine, or phenol:butanol-acetic acid solvents.

2. Elementary analyses of carbon and hydrogen agree closely with that of histidine. (Found: C = 46·30%; H = 6·07%; N = 27·15%; Theoretical: C = 46·44%; H = 5·85%; N = 27·09%.)

3. Histidine melted at 260–5° C. on a melting-point block; the sample isolated from the banana melted at the same temperature and was unaffected by a mixture with pure histidine.

4. By the potassium bromide disc method, infra-red absorption spectra were prepared from commercial *l*-histidine (Nutritional Biochemicals) and from the isolated material (Fig. 1). There is no doubt that the crystalline material isolated from the banana fruit was pure histidine in the form of its free base.

5. The substance reacts on the paper with diazotized *p*-anisidine, after the method of Jepson and Smith (1953), in the same manner as histidine.

The above evidence establishes histidine as an important free amino-acid in the soluble nitrogen of the banana fruit pulp.

Some unidentified substances in the banana[1, 2, 3]

In addition to the readily identified amino-acids and volatile amines which are described in Table 1, the chromatograms revealed the presence of twenty unidentified substances which gave colour reactions with ninhydrin on paper. These substances do not correspond to any readily identifiable ones, nor, at the present, to any of the large number of unidentified substances that have already been described from other plants (Steward *et al.*, 1955).

These substances are characterized by their position on chromatograms and by their colour reaction. A ready means of designating and classifying these is needed which should avoid confusion with substances of this category from other plants. The general system adopted in the tables and on the chromatographic maps is as follows:

Following the method of Steward *et al.* (1955), substances identified will be indicated in the tables or on the chromatographic maps by name, or by a number without a prefix, but for which a key is furnished. Unidentified compounds in the banana plant (*Musa acuminata*) which react with ninhydrin are designated MN-1, MN-2, &c.; those which react with Ehrlich's reagent ME-1, ME-2, and so on. (The prefix M for *Musa* avoids confusion with substances from other plants, and the prefix N or E, &c., indicates the reagent used or the class of compounds.)

The available information of the ninhydrin-reactive, but as yet unidentified, substances that have been recognized in the banana plant, is contained in Table 2 and in the chromatographic maps which are represented at Figs. 2 and 3. A considerable number (up to 13) of these occur in the banana plant under normal conditions of nutrition; about 9 others occur only under conditions of nutritional imbalance. Special interest attaches to certain as yet unidentified ninhydrin reactive substances which occur in the exudate that emerges from the cut shoot of the banana. These substances (designated MN-17, 18, 19, 20, Table 2) were recognized in the following way:

Chromatograms of the exudates from banana pseudostems showed trace amounts only of the more usual amino-acids (alanine, glutamic acid, serine, &c.). The only familiar amino-acid present in greater than trace amounts occupied the general position of γ-aminobutyric acid. However, in addition to the familiar amino-acids, four unidentified ninhydrin reactive materials (see Table 2) were found. The precise origin of these substances cannot be stipulated, since the exudate may include fluid from xylem, phloem, and the latex system, &c.

Having appreciated the fact that the exudate from Gros Michel banana plants contains at least four prominent, but unidentified, substances, an attempt was made to leach these from paper chromatograms and to determine their characteristics with the spectrophotofluorometer. This was to obtain

[1] The unidentified substances in the fruit were recognized in the work of Hulme.
[2] The unidentified substances in the leaf were recognized in the work of Freiberg.
[3] The unidentified substances in the exudate were recognized in the work of Freiberg, Hegarty, and Barr.

Table 2

Unidentified Substances on Chromatograms Sprayed with Ninhydrin in the Banana Plant (Gros Michel)

Unidentified no.	Rf values[1] Phenol-water pH 5·0–5·5	Collidine-lutidine (1:3) –water	*n*-butanol-acetic acid (9:1)–water	Ninhydrin colour reaction	Source
MN-1	0·809	0·346	0·108	Violet	Leaf
MN-2	0·757	0·359	0·059	Violet	Leaf
MN-3	0·679	0·385	0·131	Violet	Leaf, fruit pulp
MN-4	0·242	0·108	0·071	Violet	Leaf (potassium deficient)
MN-5	0·668	0·246	0·044	Violet	Leaf
MN-6	0·571	0·368	0·147	Violet	Leaf (phosphorus deficient)
MN-7	0·376	—	0·116	Violet	Leaf (potassium and magnesium deficient)
MN-8	0·696	—	0·116	Violet	Leaf (potassium deficient)
MN-9	0·242	0·260	0·039	Pink[2]	Leaf, fruit pulp
MN-10	0·819	0·582	0·204	Grey-violet	Leaf
MN-11	0·166	—	0·087	Pink[2]	Leaf (phosphorus deficient)
MN-12	0·357	0·488	—	Violet	Leaf (calcium deficient)
MN-13	0·487	0·243	—	Violet	Leaf (calcium deficient)
MN-14	0·265	—	0·287	Violet	Fruit pulp
MN-15	0·808	—	0·028	Violet	Fruit pulp
MN-16	0·283	0·130	0·036	Violet	Fruit pulp
MN-17	0·60	0·56	—	Yellow	Pseudostem exudate
MN-18	0·60	0·63	0·358	Brown	Pseudostem exudate
MN-19	0·66	—	—	Yellow	Pseudostem exudate
MN-20	0·84	0·59	0·525	Violet	Pseudostem exudate
M-21[3]	0·888	—	0·120	None	Leaf (K, Mg, and P deficient)
M-22[3]	0·936	0·523	0·139	None	Leaf (potassium deficient)
M-23[4,1]	1·39	—	2·26	None	Fruit pulp
M-24[4,1]	1·20	—	2·69	None	Fruit pulp
M-25[3,1]	1·46	—	3·21	None	Fruit pulp

[1] Values calculated with references to alanine as internal standard (alanine = Rf Phenol 0·59, Rf Collidine-lutidine 0·30, Rf Butanol-acetic acid 0·11).

[2] Fluoresces pink under u.v. lamp.

[3] Fluoresces blue under u.v. lamp.

[4] Fluoresces white under u.v. lamp.

information which would aid further isolation and characterization of the substances and to assess the utility of this new instrument in this sort of work.

An amount of the alcohol extract described in Part I, and which was equivalent to 5·9 g. of the original dry material, was re-evaporated to dryness

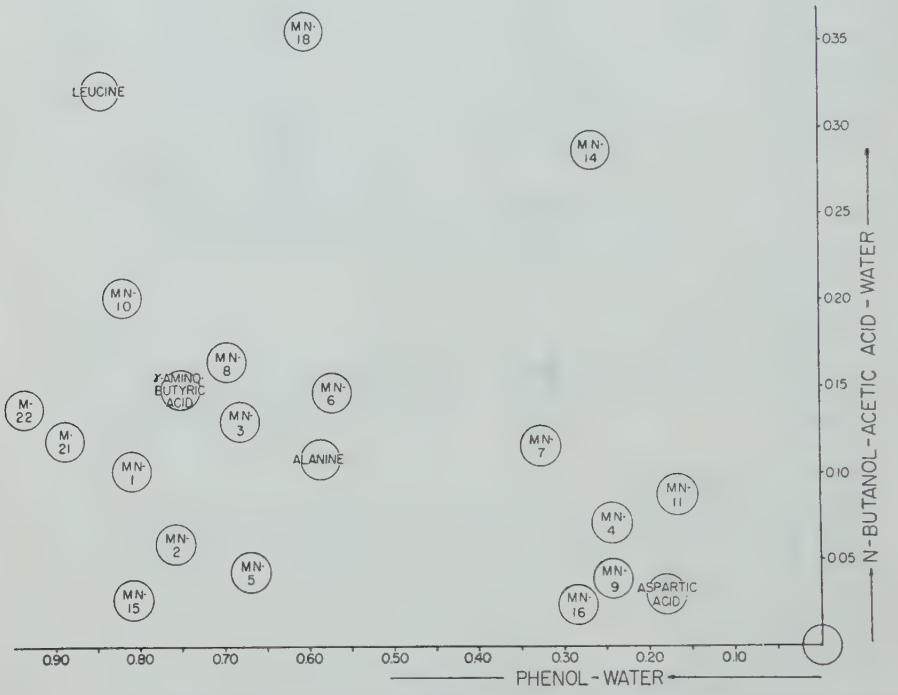


FIG. 2. Map showing position of unidentified substances in the banana plant (Gros Michel) on phenol: acid-butanol chromatograms sprayed with ninhydrin.

and dissolved in 5 ml. of distilled water. Chromatographic papers were then specially prepared by exhaustive pre-washing with butanol-acetic acid-water. Several 10 μl. aliquots were placed along the redried paper and allowed to move in butanol-acetic acid-water until the solvent front reached the edge of the paper. The two outermost chromatograms were then developed with ninhydrin to reveal the positions of the unidentified substances. From this, the corresponding spots on the adjacent untreated chromatograms could be located. These regions of the paper were removed, the areas containing the unidentified substances eluted with 4 ml. of glass-distilled water, and the extracts filtered through sintered glass to remove all traces of lint, dust, &c. The filtered extract was then used for further tests as follows:

The tests of fluorescence were made on 0·5 ml. of the filtered solution and for each test this was diluted with a further 0·5 ml. of a suitable buffer. To determine the pH response of the fluorescence, the buffers were varied to give

pH 1·3, 3·0, 5·0, 7·0, and 11·0. Two of the unidentified substances, namely MN-18 and MN-20, showed appreciable fluorescence in the ultraviolet, and the further work was confined to these substances.

In this fluorescence technique one first locates the wavelength of the

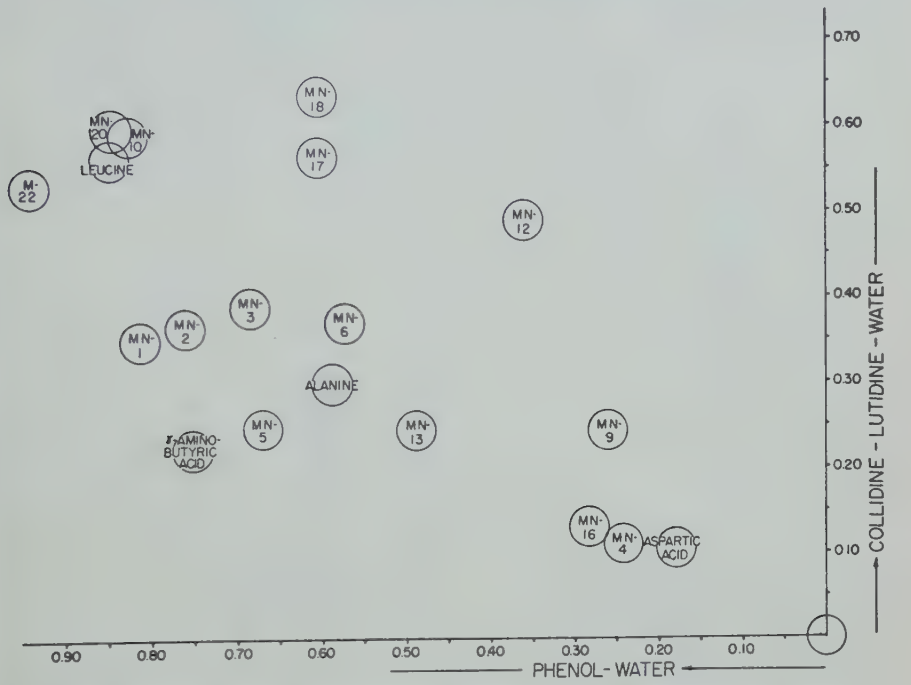


Fig. 3. Map showing position of unidentified substances in the banana plant (Gros Michel) on phenol: collidine-lutidine chromatograms sprayed with ninhydrin.

incident radiation at which the fluorescence is emitted. The instrument is then set so that the wavelength of the incident radiation remains constant for a given series of measurements. Thereafter at each pH of the buffer the instrument records the wavelength and relative intensity of the emitted radiation. The tracings obtained for MN-18 and MN-20 are shown in Fig. 4.

The characteristic responses are so sensitive and exhibit so sharp a wavelength maximum that this technique would obviously be of great value in following the isolation and purification of these substances or in determining their presence in extremely small amounts. Since the substances have not been recognized hitherto, either on other chromatograms of banana extracts or on the chromatograms of many other plant extracts, they may prove to be specific for this banana exudate material.

Fluorescent substance

In addition to the above, certain substances appear on amino-acid chromatograms with ninhydrin, but which are detectable by their fluorescence under

the ultraviolet lamp. These substances are designated M-21 and M-22 in Table 2.

A method of treating the alcohol extract of the tissue which is described later (see p. 101), with special reference to the detection of 5-hydroxy-

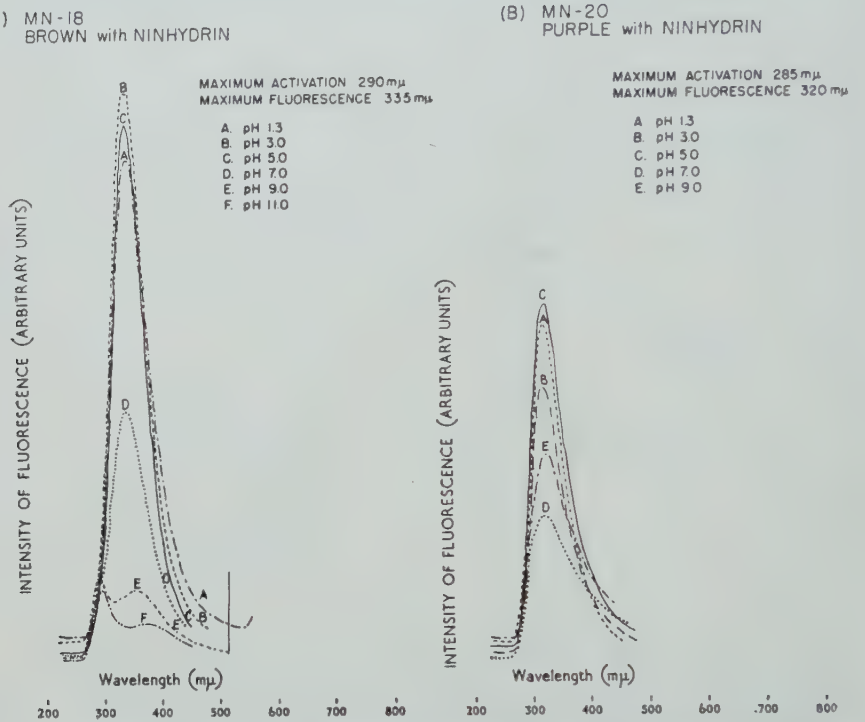


FIG. 4. Behaviour in the Aminco Bowman Spectrofluorometer of substances MN-18 and MN-20 in banana exudate from Gros Michel.

tryptamine, is particularly suitable for the demonstration of these fluorescent compounds. In this manner, compounds M-23 to M-25 were detected in the fruit pulp, and the data on these are in Table 2.

Other Nitrogenous Compounds in the Banana Plant

By treating chromatograms of the appropriate extracts with different spray reagents, still other nitrogenous compounds can be detected. The two main classes to which reference will be made are (*a*) Ehrlich's reactive compounds, including urea and certain ureides, and (*b*) guanido compounds.

A. *Substances which react with Ehrlich's reagent.*[1]

Extracts made from fruit, leaves, and root of the banana plant have been tested with this reagent, and the chromatograms reveal a variety of substances falling into the following categories:

[1] The evidence on these substances arose from the work of Freiberg and Hegarty.

(i) *Urea and ureides.* These substances react bright yellow with Ehrlich's reagent, and a map (Fig. 5) which shows the position of authentic ureides relative to urea is included to provide background material for interpretation. Urea and its ureides are now conceded to occur in plants with much greater frequency than was hitherto thought (Mothes and Engelbrecht, 1952; Bollard,

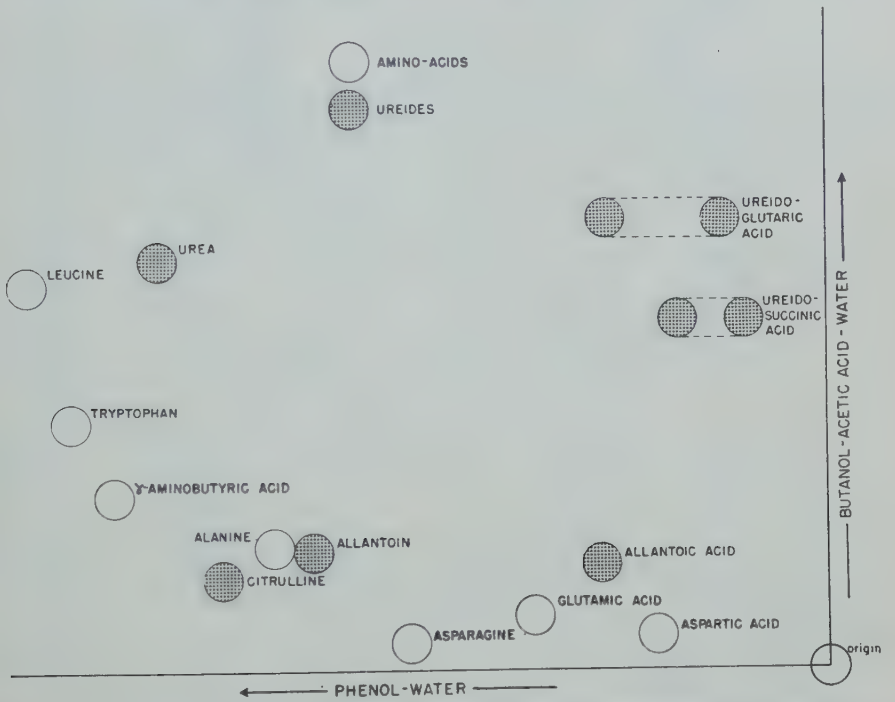


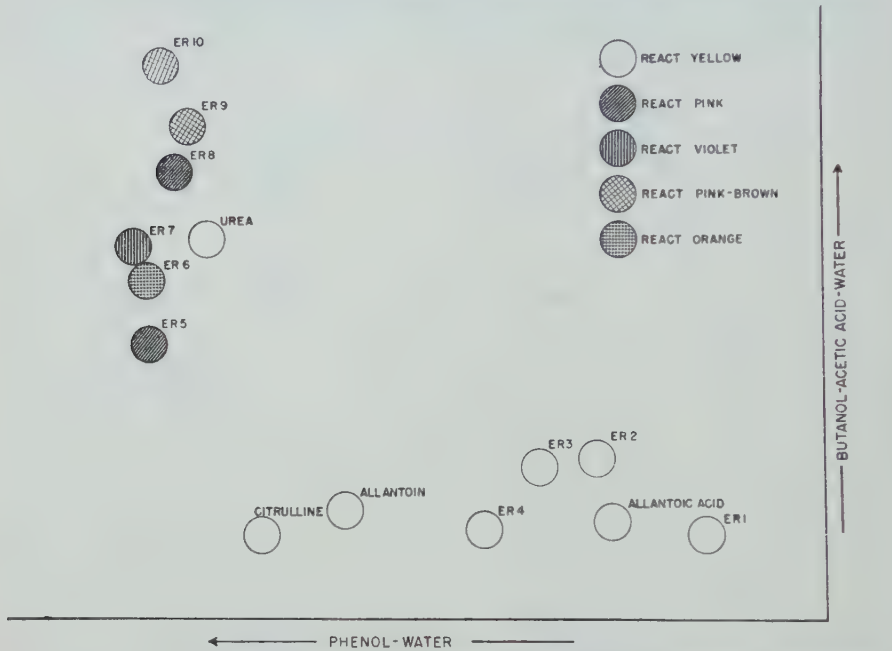
FIG. 5. Urea and some ureides and their position relative to amino-acids on phenol : acid-butanol chromatograms.

1957). In spite of the fact that urease does *not* appear to occur in the banana leaf (Freiberg and Payne, 1957), urea itself does occur, together with citrulline and the ureides allantoin and allantoic acid, and a number (10) of as yet unidentified substances. This indicates a considerable area of metabolic interest around the urea and urea derivatives that occur in the banana plant. This information will be found documented in Table 3 and Fig. 6.

The purpose of the present discussion is to indicate the range of compounds that are encountered in the banana plant (and which react with Ehrlich's reagent), even though some of these only appear conspicuously under abnormal nutritional conditions. In a subsequent paper, their relation to the particular nutritional stages and development of the plant will be discussed at greater length.

(ii) *Hydroxytryptamine and related compounds.* 5-Hydroxytryptamine reacts with Ehrlich's reagent to give a blue colour and also with ninhydrin (purple). It also has a characteristic behaviour in the spectrofluorometer (Bowman

et al., 1955). Recently, Waalkes *et al.* (1958) have detected substantial amounts of 5-hydroxytryptamine in the tissue of banana fruit and pointed to the interest of this substance because of its conversion in the animal body to 5-hydroxyindole-acetic acid. Whereas the 5-hydroxyindole compounds are commonly regarded as constituents of the animal body they have not hitherto been recognized as widely occurring in plants.



The ER numbers in this figure correspond to the ME numbers in the standard notation in Table 3.

Allantoic Acid = Prominent in young K Def. leaves, healthy and Def. leaves, trace in young fruit.
Allantoin = Severely K Def. leaves, pre-emerged fruit and pulp 61 days or older.
Citrulline = Very prominent in P Def. leaves, healthy and K Def. leaves.
Urea = Prominent in young K Def. leaves and young peel, present in most tissues.
ER1 = Healthy, P8 Mg Def. leaves and pre-emerged fruit.
ER2 = Severely K Def. leaves.
ER3 = Severely K Def. leaves.
ER4 = Severely K Def. leaves.
ER5 = Prominent in P Def. leaves, healthy K8 Co Def. leaves.
ER6 = Very prominent in pre-emerged fruit.
ER7 = Present in young fruit.
ER8 = Prominent in pre-emerged fruit, K Def. leaves.
ER9 = K Def. leaves.
ER10 = K Def. leaves.

FIG. 6. Compounds in extracts of Banana tissues which react with Ehrlich's reagent. Map showing position on phenol : acid-butanol chromatograms.

By the general method of extraction adopted in this work for nitrogen compounds, 5-hydroxytryptamine is not readily obtained on the chromatograms: this is especially true if Dowex-50 × 4 resins are used to purify the

extract, in which case only tryptophan occurs in the general area of hydroxytryptamine. In view of the work of Waalkes *et al.* (1958), the extraction procedure required to demonstrate the presence of 5-hydroxytryptamine was employed, namely that of Udenfriend *et al.* (1955), and the positive detection was verified.

TABLE 3

Compounds in Alcohol Extracts of Banana Tissue (Gros Michel) which React with Ehrlich's Reagent (2% p-dimethylaminobenzaldehyde)

	Rf value				
Identified	Phenol-water pH 5·0–5·5	*n*-butanol acetic acid (9:1)–water	Collidine-lutidine (1:3)-water	Ehrlich's colour reaction	Source
Allantoic acid	0·237	0·093	—	Yellow	Leaf, fruit
Allantoin	0·545	0·158	0·623	Yellow	Fruit pulp
Citrulline	0·637	0·079	0·228	Yellow	Leaf
Urea	0·701	0·359	0·497	Yellow	Leaf, fruit
Unidentified					
ME-1	0·127	0·075	—	Yellow	Leaf, fruit
ME-2	0·254	0·149	—	Yellow	Leaf (K deficient)
ME-3	0·326	0·142	—	Yellow	Leaf (K deficient)
ME-4	0·389	0·081	—	Yellow	Leaf (K deficient)
ME-5	0·770	0·257	—	Pink	Leaf
ME-6	0·770	0·318	—	Orange	Pre-emerged fruit
ME-7	0·784	0·352	—	Violet	Fruit
ME-8	0·735	0·427	—	Pink	Leaf (K deficient, Pre-emerged fruit)
ME-9	0·722	0·466	—	Pink-brown	Leaf (K deficient)
ME-10	0·756	0·521	—	Pink-brown	Leaf (K deficient)

In order to obtain unequivocal evidence of the presence of 5-hydroxytryptamine on amino-acid chromatograms of the banana, the following procedure is adopted. The concentrated alcohol extract is dissolved in water and partitioned between water and phenol-water at pH 5·5. The phenol layer containing the 5-hydroxytryptamine and most of the amino-acids is evaporated and, at an appropriate concentration, used for the two-directional chromatograms. The 5-hydroxytryptamine is detected on the paper by reaction with an 0·2 per cent. solution of ninhydrin in acetone mixed with glacial acetic acid in the ratio 9:1 (see Smith, 1958). The 5-hydroxytryptamine appears as a yellow-brown spot in visible light between pipecolic acid and valine on phenol : butanol-acetic acid chromatograms, but, viewed by ultraviolet radiation, a very sensitive greenish fluorescence appears. As it occurs in banana extracts, 5-hydroxytryptamine is accompanied by some substance that reacts, on standing, to give an orange-brown colour with Ehrlich's reagent: this substance appears directly below 5-hydroxytryptamine on phenol : methyl, ethyl ketone-formic acid-water (50:4:10) chromatograms.

B. *The guanido compounds*[1]

Other than arginine, little attention has been paid to soluble nitrogen in plants which may appear in the form of guanido compounds. To provide background material to interpret these, a chromatographic map has been pre-

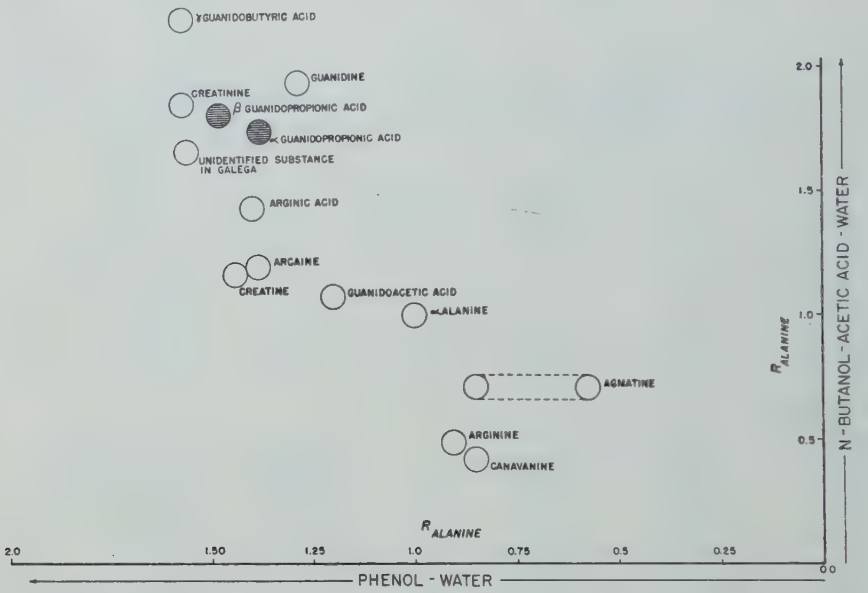


FIG. 7. Map showing the positions of some substituted guanidine compounds on phenol: butanol-acetic acid chromatograms.

○ Substances known to occur in nature

◍ Substances as yet unknown in nature

pared for a number of authentic guanido compounds (Fig. 7). By the use of the extended ninhydrin method already described, three positively reacting areas were noted on the chromatograms. Two of these are due to sugars. The third substance which reacts positively with the extended ninhydrin test occurs in the chromatographic position of γ-guanidobutyric acid. This substance also reacts positively with the Sakaguchi reagent and with alkaline nitroprusside, both reagents for monosubstituted guanidine compounds. On the basis of colour reactions on the paper and on the basis of chromatographic position, this substance would seem to be γ-guanidobutyric acid as reported by Irreverre *et al.* for banana (1957). However, this substance completely disappears under conditions of mild alkaline hydrolysis which have no effect whatsoever on added γ-guanidobutyric acid. For this reason the substance is thought *not* to be γ-guanidobutyric, and the problem of its actual identity must await actual isolation and critical identification.

[1] The evidence on these substances arose from the work of Pollard and Hegarty.

FIG. 8. Chromatogram of organic acids of edible banana.

The Keto-Acids and the Organic Acids of the Banana Fruit

A. *Keto-acids*[1]

The fruit of the banana contains numerous keto-acids. Some of these could immediately be recognized and some remain unidentified. Table 4 lists the identifiable keto-acids of the banana pulp together with the amino-acids by which they were recognized. Of the ten keto-acids so listed, three (oxaloacetic, α-ketoglutaric, and pyruvic) are such general metabolic constituents of plants that their occurrence in the banana need occasion no surprise. At the

[1] The evidence on the keto-acids arose from the work of Rabson.

stage of harvest and prior to ripening, pyruvic acid predominated; but as ripening proceeds, the relative proportion of these substances changes in ways that will be described in a later paper.

Glyoxylic acid, giving rise to glycine, is again a substance, the occurrence of which has previously been well established both by isolation and the fact that it plays a part in known plant enzyme systems (Tolbert *et al.*, 1949).

In the analytical procedure both α-ketoglutaric acid and succinic semialdehyde give rise to γ-aminobutyric by the technique here used, but the amount of γ-aminobutyric acid which arises from α-ketoglutaric acid by decarboxylation is small. Therefore, the occurrence of γ-aminobutyric acid on the final chromatograms in amounts larger than would be expected by the decarboxylation of α-ketoglutaric acid during the analytical process is presumptive evidence for the occurrence of succinic semialdehyde.

Two other keto-acids which may be tentatively identified, namely α-keto-isovaleric acid (dimethylpyruvic) and β-hydroxypyruvic acid, which are recognized as valine and serine respectively, have been reported to occur in *Tulipa* and *Allium* respectively (Towers, Thompson, and Steward, 1954). The occurrence of the last three keto-acids of Table 4, though probable, is still tentative.

In Table 4 there are data which designate twelve acidic carbonyl compounds that were recognized on the chromatograms as their amino-acid analogues, though the latter did not correspond to known substances. Some of these substances, notably MK-1 and 5, were prominent constituents of the keto-acid fraction of the unripe harvested fruit.

From the number of keto-acids which are at least tentatively identified and the number which are recognized but are still unidentified, it is clear that there is need for a more systematic investigation of this class of compounds in this and other plant materials.

B. *Non-volatile organic acids*[1]

A preliminary qualitative chromatographic survey of the organic acids present in the banana plant was made on the extracts prepared from the pulp of fruit as described earlier in this paper. A typical chromatogram made with benzyl alcohol, tertiary butanol, isopropanol, formic acid, water, 3:1:1:0·125:1 (Stark, Goodban, and Owens, 1951), is illustrated in Fig. 9. This figure shows that at least ten detectable acids are present in the pulp of banana fruit. By extending this kind of one-directional chromatographic procedure to three different solvent systems, and by comparing the organic acids of banana with known organic acids, citric and malic acids could be identified in the extracts, and the possible presence of glycollic and quinic acids indicated.

After this survey had indicated the number of organic acids which are present in banana fruit, they were separated by a process which combined the

[1] The evidence on the organic acids arose from the work of Hulme.

TABLE 4

The Keto-Acids of the Banana Fruit Pulp (Gros Michel)

Keto-acid	Amino-acid product of the analytical process
Identified	
α-ketoglutaric acid	Glutamic acid, γ-aminobutyric acid (and CO_2)
Pyruvic acid	Alanine
β-hydroxypyruvic	Serine
α-ketoisovaleric	Valine
Succinic semialdehyde	γ-aminobutyric acid
Glyoxylic acid	Glycine
Oxalacetic acid	Aspartic acid, α-alanine (and CO_2), β-alanine
α-ketoisocaproic acid and/or	Isoleucine
α-keto, β-methylvaleric	Leucine
α-keto, β-hydroxypyruvic acid (?)	Threonine

Unidentified	R_f values[1]	
	phenol-H_2O	butanol-acetic acid-water
MK-1	0·055	0·020
MK-2	0·445	0·050
MK-3	0·525	0·070
MK-4	0·645	0·104
MK-5	0·865	0·280
MK-6	0·620	0·044
MK-7	0·400	0·100
MK-8	0·510	0·095
MK-9	0·275	0·110
MK-10	0·835	0·147
MK-11	0·935	0·236
MK-12	0·785	0·276

[1] R_f values for α-alanine in these solvents are phenol-water, 0·59 and butanol-acetic acid-water, 0·11.

gradient elution method of Hulme and Wooltorton (1958), with chromatographic examination of the fractions as they were obtained by this gradient elution process. The results are shown in Fig. 9 and Table 5.

Eleven identifiable organic acids have now been found in the banana fruit; nineteen remain still to be identified. The bases for establishing the identity of the eleven acids are summarized in Table 5. On this evidence, malic and citric acids are the predominant organic acids of the banana fruit pulp. Of the remaining acids, none appear in comparable amounts to citric and malic, although the third most abundant acid, i.e. # 1 of Fig. 9, is still unidentified. The occurrence of pyroglutamic (pyrrolidone carboxylic acid) may be an artifact due to decomposition of glutamine during extraction or the ion-exchange purification of the extracts. The analytical method was only able to demonstrate succinic, malic, and citric acid of the well-known organic acids of the Krebs cycle, but the principal keto-acids of the cycle (pyruvic, oxalacetic, α-ketoglutaric) had already been found (see Table 4); aconitic, cis-aconitic, fumaric, oxalsuccinic, and isocitric acid were undetected. It is possible that

TABLE 5

Some Details of the Organic Acids Found in Bananas

Acid number (see Fig. 9)	RMA (Solvent 1)	Identity	Reactions $AgNO_3/NaOH$[1]	Ferrocyanide reagent[2]	Permaganate and Na_2CO_3[3]	Other tests and remarks
1	0·15	—	−ve	Light green	−ve	Blue fluorescence in u.v. light.
2	1·37	Lactic	−ve	−ve	−ve	Probably an artifact from sugar by the action of the basic resin then used.
3	0·15	—	+ve	Light blue-green	−ve	Test[1] immediate, therefore probably a sugar acid.
4	1·63	—	−ve	−ve	−ve	Only present in traces.
5	0·61	Shikimic	+ve	Light green	+ve	Positive Roberts and Cartwright test.[4]
6	0·28	—	+ve	−ve	+ve	—
7	0·52	—	+ve	Blue grey	−ve	—
8	1·59	—	−ve	−ve	−ve	—
9	0·30	—	+ve	−ve	−ve	—
10	0·17	—	+ve	Blue grey	−ve	—
11	0·35	Quinic	+ve	Blue grey	−ve	Positive Roberts and Cartwright test.[4]
12	1·54	—	−ve	−ve	−ve	—
13	0·44	—	+ve	Blue grey	−ve	Test[1] immediate, probably a sugar acid.
14	1·17	Glycollic	−ve	Blue grey	−ve	—
15	0·76	Glyceric	+ve	Blue grey	−ve	Blue colour H_2SO_4 and α-naphthoresorcinol.
16	0·11	—	+ve	Blue	−ve	Test[1] immediate, probably sugar acid.
17	0·22	—	+ve	−ve	−ve	—
18	1·10	Pyroglutamic	−ve	Duck egg green	−ve	Pyrrolidone carboxylic acid, probably an artifact from glutamic due to the extraction procedures.

19	1·54	Succinic	−ve	Duck egg green	−ve	—
20	1·71	—	−ve	−ve	−ve	—
21	0·17	—	+ve	Blue grey	−ve	Test[1] immediate, probably sugar acid.
22	0·39	—	+ve	Blue grey	−ve	Test[1] immediate, probably sugar acid.
23	0·55	—	+ve	−ve	−ve	—
24	0·11	—	+ve	−ve	−ve	—
25	1·28	Citramalic	−ve	Blue	−ve	—
26	1·00	Malic	−ve	Deep blue	−ve	—
27	1·46	—	−ve	Blue grey	−ve	—
28	1·70	—	−ve	Blue grey	−ve	—
29	0·50	Tartaric	+ve	Purple	−ve	Red colour with ammonium vanadate.
30	0·80	Citric	−ve	Deep blue	−ve	—

[1] This test is described by Anet and Reynolds (*Australian J. Chem.*, 1955). 'Sugar acids' give an immediate black colour; glyceric, keto-acids, and quinic and shikimic acids give a dark brown colour on standing in the cold.

[2] This test is described by Martin (1955). We have found that the only acids giving pronounced reactions (we did not try oxalic) are pyroglutamic (pyrrolidone carboxylic) acid, fumaric and succinic acids which give a pale green (first stage of Martin's treatment) and a strong duck egg green (second stage).

[3] This is a test for reducing groups.

[4] This test (Cartwright and Roberts, 1955) which is a metaperiodatenitroprusside test appears to be specific for quinic, shikimic, dihydroshikimic, and, presumably, similar acids.

failure to detect the aromatic acids may be attributed to the charcoal treatment used in the preparation of the extracts. Though oxalic acid is known to occur in banana (Kohman, 1939), the unidentified (No. 21 or 24) dibasic acids (see Fig. 9) are *not* identical with oxalic acid.

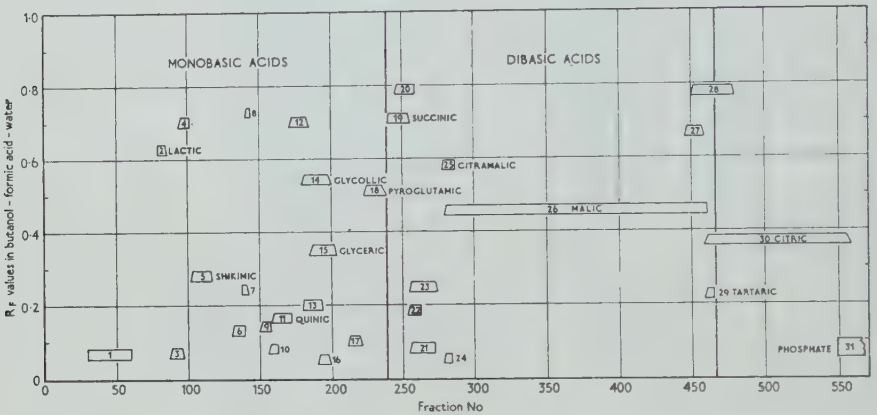


FIG. 9. Separation of the organic acids of the ripe banana (Gros Michel) pulp

Ordinates show one-directional paper chromatograms (butanol-formic acid-water) of fractions eluted from a column.

Abscissae record by No. the fractions eluted from the column.

Identified acids indicated by name & No. and unidentified by No. only (see Table 5).

In view of the widespread occurrence of quinic acid in plants, its occurrence in the banana fruit pulp is not surprising. However, the occurrence of the similar but partially unsaturated shikimic acid which even in 1950 had only limited distribution (Bonner, 1950) is more noteworthy. The lower and volatile fatty acids (such as acetic and butyric) which are known to occur in the banana (von Loesecke, 1929) were not, of course, detected in this work.

Hydrolytic products of the alcohol-insoluble fraction[1]

All the preceding data have been assembled from the fractionation of the alcohol-soluble materials of the banana plant. The alcohol-insoluble residue contains the protein, and therefore the detection and determination of the amino-acids in the hydrolysates reflects the composition of the protein *in toto*. Data of this kind are assembled in Table 6.

As to be expected, the leaf contains the largest amount of total protein per unit fresh weight of tissue, having nearly ten times the protein content of fruit and root. The total protein present in these three different organs have different relative amino-acid compositions. The protein of the fruit is particularly rich in dicarboxylic acids, in the leucines, and contains more histidine than the protein of other regions; but it is relatively deficient in arginine and in the aromatic amino-acids. In the proteins of the leaf and root,

[1] The evidence on the hydrolytic products arose from the work of Hulme and Freiberg.

TABLE 6

Amino-acid Composition of the Alcohol Insoluble Protein Hydrolysates in the Banana Plant (Gros Michel)

Amino Acid[1]	Leaf[2]	Fruit[3]	Root[4]
Aspartic-acid	+	++++	+++
Glutamic acid	+++	++++	++
Serine	++	+	++
Glycine	++	++	++
Threonine	++	+	++
Alanine	++	++	++
Histidine	—[5]	+	—[5]
Lysine	++	++	++
Arginine	++	+	++
Methionine sulfoxide	+	—[5]	—[5]
Proline	+++	++	+++
Valine	++	++	++
Leucine(s)	+++	++++	+++
Phenylalanine	+	—[5]	++
Tyrosine	++	+	++
Total amino-acids m./g. fresh wt.	22·70	3·13	2·86

[1] When the specified amino-acid represents less than 5% of total amino-acids = +, 5 to 10% = ++, 10 to 15% = +++, greater than 15% = ++++.

[2] Second youngest fully expanded banana leaf of plant growing in sand culture in Honduras.

[3] Fruit pulp at harvest time in the tropics and ready for shipment.

[4] Young actively growing roots from banana plants grown in sand culture at Cornell University.

[5] Present but below the level of chromatographic determination.

glutamic acid, proline, and the leucines are the major amino-acid components, aspartic acid being notably low in the leaf protein through a dominant constituent in the root.

SUMMARY

1. A survey has been made of various constituents of the banana plant (*M. acuminata* cv. Gros Michel). Use has been made of the techniques of chromatography to separate and identify these constituents. The following parts of the plant have been examined: leaf, leaf sheath (the rolled, basal portion of the leaf which extends for the length of the pseudostem), fruit ('pulp' and 'peel' at harvest, young ovaries and styles during development), rhizome and root (cortex and stele); and in addition the exudate from cut shoots has been examined.

2. The techniques applied revealed the following categories of substances on the chromatograms: non-volatile and volatile soluble-nitrogen compounds which react with ninhydrin; compounds which react with *p*-dimethylamino-benzaldehyde or Ehrlich's reagent (urea, ureides, indole compounds, and certain phenolic substances); certain compounds (guanido derivatives) detectable with ninhydrin followed by alkali; keto-acids detected by ninhydrin

after conversion to amino-acids; and non-volatile organic acids detectable by indicator (bromocresol green).

3. The non-volatile alcohol-soluble nitrogen compounds comprise the following:

The prominent identified amino compounds are aspartic acid, glutamic acid, asparagine, glutamine, alanine, arginine, histidine, γ-aminobutyric, and pipecolic acid. The leaf is distinguished by emphasis on glutamic acid, alanine, and γ-aminobutyric acid, and by the relatively low content of glutamine, which is conspicuous elsewhere, and also by the absence of pipecolic acid, which occurred in all other portions of the plant which were examined. The fruit, on the other hand, is relatively rich in the amides and in histidine in the fruit pulp. Aspartic acid and γ-aminobutyric acid and pipecolic acid are also prominent in the pulp of the mature fruit. Arginine is abundant in the more actively growing regions (e.g. the leaf sheath, young ovaries and styles, and young roots). Glutamine is the main nitrogenous storage product of the rhizome and mature roots.

In addition, a total of twenty unidentified ninhydrin reactive substances were detected and their characteristics noted. Some of these were prominent under conditions of nutritional imbalance, and some were conspicuous in the exudate from cut shoots.

4. Three volatile nitrogen bases were detected in the leaves, two were identified as ethanolamine and putrescine, and the third is still unidentified.

5. The identifiable compounds detected with Ehrlich's reagent were allantoic acid, allantoin, citrulline, and urea; but in addition ten unidentified substances were recorded; of these probably four were ureido compounds reacting yellow, and the others may be either of an indolyl or phenolic nature. Some of these were only prominent under conditions of potassium deficiency or special development conditions.

6. Arginine is the prominent guanido compound, but one other such compound also occurs prominently. Although this has been referred to as γ-guanidobutyric acid, this is now open to some question.

7. The acidic carbonyl compounds which were recognized after conversion to the amino acids comprise the following prominent keto acids, α-ketoglutaric acid, pyruvic acid, β-hydroxypyruvic acid, α-ketoisovaleric, and succinic semialdehyde. In addition oxalacetic and glyoxylic acids occurred in smaller detectable amounts, and several other keto-acids were postulated from their amino-acid analogues. Thirteen other acidic carbonyl compounds gave rise to amino compounds not yet identified; of these, five were prominent in the fruit.

8. The non-volatile organic acids of the banana pulp were investigated by the combined techniques of elution from columns and paper chromatography.

The most prominent identified organic acids were malic and citric acids, but in addition citramalic and succinic also occur. Glycollic and glycine were prominent, tartaric acid was present, and acids related to inositol (quinic and

shikimic) were also found. In addition nineteen unidentified acidic substances appear in the chromatograms.

9. The amino-acid compounds of the total protein present in leaf, stem, and root has been determined, and certain differences between them are noted.

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Physiological Investigations on the Banana Plant

II. Factors Which Affect the Nitrogen Compounds of the Fruit

BY

F. C. STEWARD, S. R. FREIBERG,[1] A. C. HULME,*[2]
M. P. HEGARTY,†[3] R. BARR,‡ AND R. RABSON§[4]

(*Department of Botany, Cornell University, Ithaca, New York*)

With seven Figures in the Text

ABSTRACT

A quantitative study has been made of the way in which the nitrogenous compounds which are present in the banana fruit are affected by the changes that ensue subsequent to harvest. This was done with special reference to *Musa acuminata* cv. Gros Michel as grown in Central America (Part A), but comparative data are furnished for the cultivars and for material from other areas of cultivation (Part B). In the cultivar Gros Michel from Honduras, the amides (asparagine and glutamine) and histidine play a predominant role in the soluble nitrogen fraction of the fruit. Prominent changes occur in the nitrogen (N) compounds as the fruit ripens, and these are shown to be markedly influenced by the conditions that obtain during the development of the fruit. The nitrogenous composition of the fruit (Gros Michel) is markedly affected by the season of the year at which it forms (Part C); this affects the balance between the amides (asparagine and glutamine) and histidine, and also influences the post-harvest metabolic behaviour in ways which are described. While the total protein of the fruit is relatively stable in its amino-acid composition, certain differences are noted which seem to characterize the cv. Gros Michel as it grows in Central America, and some shifts in the amino-acid balance in the protein do seem to occur with ripening.

A typical growth curve for the parthenocarpic fruit of *M. acuminata* cv. Gros Michel is given, and the changes in the N compounds that occur during development (Part D) are related to this and to the seasonal effects referred to in Part C.

The very young inflorescence is first relatively rich in soluble N compounds. After the early phase of growth (cell division) has elapsed, the fruit utilizes its soluble N in synthesis faster than it is supplied to the fruit, so the soluble N complement falls to a low level; thereafter it increases again to a new higher level at harvest. The composition of the soluble N fraction as stored at harvest is quite different from that which obtains in the inflorescence in which the fruit is being

* The work of Hulme relates particularly to Part A.
† The work of Hegarty relates particularly to Part D.
‡ The work of Barr relates particularly to Parts B, C, and E.
§ The work of Rabson relates particularly to Part A (data on keto-acids).

Present address: [1] United Fruit Co., Central Research Laboratories, Upland Road, Norwood, Mass., U.S.A.
[2] Ditton Laboratory, Larkfield, Maidstone, England.
[3] Plant and Soils Laboratory, C.S.I.R.O., Brisbane, Australia.
[4] Biology Dept., University of Houston, Houston, Texas, U.S.A.

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initiated. The latter emphasizes the amides (asparagine and glutamine) and histidine; the former emphasizes alanine and glutamine as the only prominent amide.

The trend of events is markedly affected by the time of year when the fruit develops—development in the winter leading to a low amide (especially glutamine)/high histidine condition; whereas development in the summer leads to a high amide (asparagine and glutamine)/low histidine condition at the normal time of harvest. In the more rapid development of summer-grown fruit, certain changes in N compounds occur after harvest, which in the slower developing winter-grown fruit precede the harvest.

The different regions of the fleshy fruit, i.e. the fleshy pericarp (endocarp) and the inner region composed of carpels, differ greatly in the total quantity and in the composition of the nitrogenous compounds they contain. The carpels are richer in soluble N and contain all of the amide of the ripe fruit.

Contents

PHYSIOLOGICAL observations on the banana plant were made as early as 1807 (Fourcroy and Vauquelin), but from the outset these have concentrated upon the carbohydrates present and upon the changes which are associated with respiration and ripening of the fruit. Notwithstanding the extensive studies already made (see Part I), remarkably little attention has been given to the behaviour of the nitrogenous compounds of the fruit, except to say that the protein fraction changes but little during the ripening process (Gore, 1915; Stratton and von Loesecke, 1930). The qualitative survey of the nitrogenous compounds which are to be found in the banana, which is contained in Part I, provides the background against which the quantitative changes may now be discussed.

The first objective of this paper is to show that the biochemical changes that occur during the ripening process also extend to the behaviour of the soluble-nitrogen compounds in the fruit: this is notably true of changes that concern the amides, on the one hand, and histidine, on the other. After presenting this information for both Gros Michel and other cultivars, the later part will show the effects which may be observed in the banana fruit at different stages of growth.

Experimental Methods

In this part, the method simply involved the examination of the unripened fruit, obtained at the stage at which it normally reached the port of disembarkation, and also of fruit at subsequent periods during the ripening process. This was first done for stems of Gros Michel received in Ithaca, New York, from the port of entry (New York City) from Central America,

and later from Gros Michel fruit ripened in Honduras and shipped in ethyl alcohol to Cornell University: these samples were obtained with the help of the United Fruit Company. Subsequently the same general methods were applied to other banana cultivars as they arrived in London from other centres of banana production (Gros Michel, Lacatan, Cavendish). These samples were obtained with the co-operation of Messrs. Elders & Fyffe of London, England. In this way the biochemical changes that occur in the nitrogen compounds were observed in some detail for the cultivar Gros Michel, and then the extent to which this behaviour could be regarded as general was assessed from the data that relate to other sources of supply and to other varieties.

Samples were drawn from 'hands' which were located at different levels along the floral stalk, and individual fruits were drawn from the central region of each such sampled 'hand', while the tissue actually extracted and analysed was removed from the fruit half-way along its length. This sampling procedure minimizes the effects due to variation within one stem. Each sampled fruit was cut transversely at the mid-point along its length. Adjacent to the cut so made three slices, each $\frac{1}{2}$ inch thick, were then cut from each of the severed portions. The peel was trimmed and discarded. Tissue from enough comparable 'fingers' was accumulated to assure the replicate samples were representative of the material and adequate in quantity for analysis, and the agreement between them indicated that this had been achieved. Enough alcohol was added to the freshly weighed 'pulp' to make the mixture 75 per cent., allowing for the water content of the tissue. The samples were then stored at 20° C. until they could be analysed. The alcohol-soluble material was extracted and concentrated in readiness for chromatographic analysis in the manner described in Part I. The alcohol-insoluble residue was retained for the examination of the amino-acids which are released upon acid hydrolysis of the total residual alcohol-insoluble protein.

The quantitative paper partition chromatographic procedure used was based on that described earlier by Thompson and Steward (1951), though in the interim certain details of technique had been perfected, and the method had been carefully standardized through repeated use so that accurate standard curves were available for all the amino-acids in question.

Wherever possible, the alcohol extracts were used without supplementary techniques to remove interfering substances. However, when this was not possible, as, for example, in the case of the almost fully ripened fruit, the extracts were purified by absorption upon a Dowex 50×4 column, so that the sugars, &c. were washed through while the amino-acids were first absorbed on the column and then eluted by the use of 0·15N ammonia. This was done at 4° C. and in ways which preserved such labile substances as glutamine.

Different stems of banana (Gros Michel) may ripen at different rates; in fact, two such stems received together from the same ship, when placed in a ripening chamber at 70° F. could be distinguished as a faster (Stalk A) and a slower (Stalk B) ripening stem; Stalk A reached in about 8 days a

similar condition to that attained by Stalk B in 12 days, by which time most of the fruit had reached the yellow condition designated 6 to 7 on the standard colour chart which is used for recording the stage of ripening in bananas (von Loesecke, 1950). Also by this time the peel and pulp separated readily, the tannin as revealed by the ferric chloride reaction had disappeared, and most of the starch had also disappeared. However, throughout the ripening process, analyses showed that the proportion of alcohol-soluble nitrogen to alcohol-insoluble nitrogen remained approximately constant.

TABLE 1

Nitrogen Content of Pulp of Ripe and Unripe Banana Fruits

(*Kjeldahl Method*)

State of maturity	Mg./100 g. fresh weight		% of total N	
	Sol. N	Insol. N	Sol. N	Insol. N
Green	50·5	93·5	35·1	64·9
Yellow	53·9	104·8	33·9	66·1

The alcohol-soluble nitrogen of the pulp from Stalk A was at the outset lower than that of Stalk B, and this was particularly noticeable for the amides glutamine and asparagine. Despite this difference, the trend of the changes observed during ripening will be presented for Stalk B; recognizing that it followed a somewhat similar trend in the case of Stalk A.

A. Changes in the Soluble Nitrogen Compounds of Banana Fruit (Gros Michel) during Ripening

The changes now to be described are entirely within the alcohol-soluble fraction, and they occurred with the minimum of change in the ratio of alcohol-soluble to insoluble nitrogen in the fruit (Table 1).

Table 2 records the initial analysis of the alcohol-soluble nitrogen compounds in the tissue, derived from the two stems, A and B; and it also shows the trend with time as these nitrogen compounds responded to the ripening process (Stalk B).

Prior to the onset of ripening, asparagine, glutamine, and histidine comprised the bulk of the alcohol-soluble nitrogen. This is shown in Table 2. Pipecolic acid, together with aspartic acid, stands fourth in the amount present in the tissue; this was somewhat unexpected as pipecolic acid was only recently discovered in legumes (Zacharius *et al.*, 1954) and had not previously been reported for banana tissue, although it does occur frequently in other monocotyledonous plants (Fowden and Steward, 1957). γ-Aminobutyric acid, now known to be almost ubiquitous in plants, is also a prominent constituent of the fruit.

The experimental data can be conveniently presented in two ways. Table 2 expresses the amino-acids as micrograms of acid per gramme fresh weight of

fruit pulp, and it also records the changes which occur with time (for Stalk B). Fig. 1 shows the relative composition of the soluble nitrogen in the tissue by showing nitrogen in each amino-acid as a percentage of the total soluble nitrogen, as determined by ninhydrin, which includes all the amino-acids and amides.

For comparison (and merely to recognize a different type of behaviour which will later be explained as the behaviour of 'winter-grown' fruit) Fig. 2 is included. Fig. 2 presents data on Gros Michel bananas harvested and ripened in the tropics and kept free of treatment by ethylene or known growth-regulating substances. In those cases where samples remained longer in alcohol than was normally the case, it was verified that although some

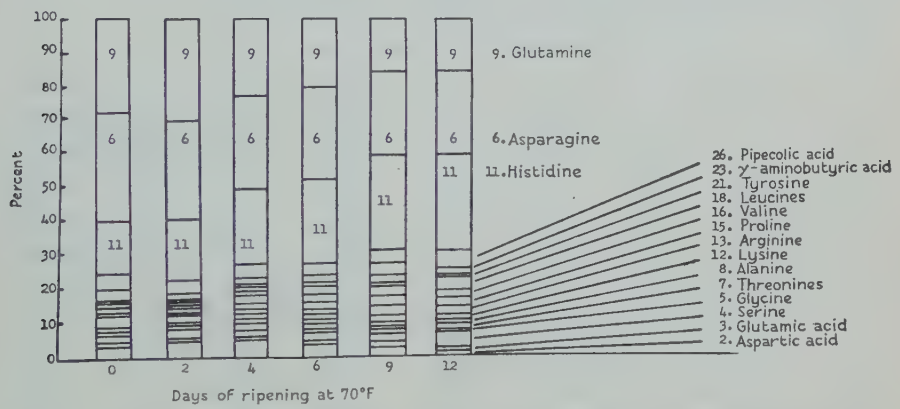

FIG. 1. Alcohol-soluble nitrogen of the fruit of the banana—changes occurring during ripening. (Nitrogen of each compound as per cent. of soluble nitrogen determined by ninhydrin.)[1]

losses of glutamine did occur under similar conditions, they were not such as to invalidate the conclusions. The behaviour of asparagine and histidine was not affected by storage of the extracts.

The conclusions that emerge from these data are as follows:

Firstly, Fig. 1 shows that there are certain soluble nitrogen compounds, the content of which tends to decrease as ripening proceeds. These are the amides asparagine and, more notably, glutamine, and the amino-acids aspartic acid and alanine. Conversely, the content of certain amino-acids, tends to increase as ripening proceeds. These are histidine, valine, and leucine(s). The main changes, however, are in the amides asparagine and glutamine which tend to decrease, and in histidine which tends to increase. Fruit grown in the period October to February, as shown in Fig. 2, contained a much smaller quantity of glutamine and a much higher initial content of histidine than fruit which was received in Ithaca, New York, and ripened in the autumn. Also, the basic amino-acids arginine and lysine and leucine and valine are prominent.

[1] Where amino-acids are designated by number, the code adopted by Dent, Stepka, and Steward, Nature, 1947, 160, 682 is followed.

Whenever glutamine appears unexpectedly low in plant samples, the possibility of its loss during the analysis by conversion to pyrrolidone carboxylic acid arises. A check was made against the possibility that the low glutamine nature of certain samples of banana fruit might be an artifact of analysis. Glutamine in solution and added to banana tissue was subjected to similar conditions of storage, extraction, and analysis. It was verified in this way that any losses of glutamine that might have occurred during extraction, storage of extracts, &c. (even though the conditions were designed to prevent this)

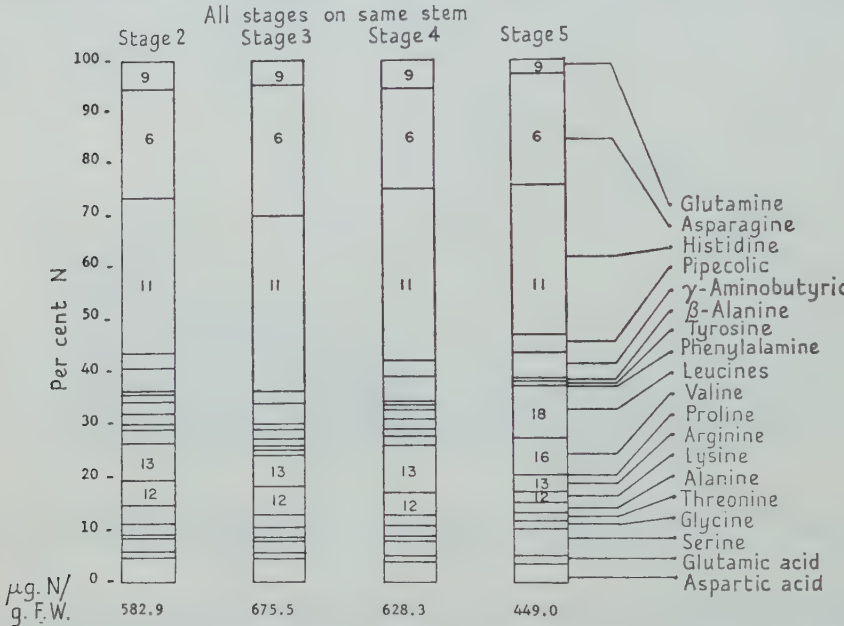

FIG. 2. Alcohol-soluble nitrogen of Gros Michel banana harvested and ripened in Honduras in February. (Nitrogen of each compound as per cent. of soluble nitrogen determined by ninhydrin.)

could not have been sufficient to affect the interpretation seriously. Furthermore, the substances asparagine and histidine, which are also involved in the interpretation, are very stable substances, and their behaviour could not even be attributed to their instability during analysis.

On the behaviour of the keto-acids during ripening[1]

Of all the non-nitrogenous metabolites, those most closely linked with nitrogen metabolism are the keto-acids: therefore, their behaviour becomes of special interest. Methods for the detection and determination of the keto-acids as their corresponding amino-acids have been mentioned in Part I of this series.

The samples of banana fruit on which the data of Table 2 were obtained

[1] The data in this section are based on work of Rabson.

were also analysed for the keto-acids: the data are presented in Table 3. The content of keto-acid is expressed as μgs. of keto-acid per g. fresh weight. These data were calculated from the corresponding quantities of the amino-acids actually determined. Recognizing the differing efficiencies of the hydrogenolysis which converts the keto-acid hydrazines to their respective amino-acids,

TABLE 2

The Soluble Nitrogen Content of the Banana Fruit: the Changes that Occur During Ripening

(*Data as micrograms amino-acid per g. fresh wt.*)

	Stalk B						Stalk A
Days of ripening	0	2	4	6	9	12	0
Amino-acid:							
Aspartic acid	13·4	16·2	12·8	8·6	9·8	10·2	13·8
Glutamic acid	3·9	3·3	3·2	2·5	7·2	1·4	2·0
Serine	6·5	7·5	5·6	6·4	8·4	12·9	3·4
Glycine	2·3	2·0	3·0	2·2	2·2	4·3	0·9
Asparagine	60·0	53·2	40·4	41·9	55·0	56·5	29·9
Threonine	2·9	3·3	3·0	3·2	3·3	5·0	1·3
α-Alanine	10·0	5·7	3·9	3·5	4·1	2·8	5·6
Glutamine	57·2	57·7	37·7	33·8	35·8	32·0	27·2
Histidine	22·5	24·8	25·3	29·7	45·6	42·5	18·4
Lysine	1·9	1·3	1·4	1·6	3·8	3·1	1·3
Arginine	2·5	trace	1·7	1·7	5·0	trace	—
Proline	2·0	5·0	5·4	6·7	10·5	7·5	3·2
Valine	1·0	2·0	3·1	4·3	7·8	7·8	2·0
Leucine(s)	1·2	2·6	5·6	8·7	15·4	14·8	4·0
Tyrosine	2·0	1·4	2·2	1·2	2·3	1·8	1·9
β-Alanine	trace	trace	trace	trace	trace	trace	trace
γ-Aminobutyric acid	12·0	6·3	6·3	7·2	12·3	8·5	6·5
Pipecolic acid	13·3	15·5	9·4	9·4	13·0	15·0	9·0

these values furnish valid comparisons even though they are not strictly absolute.

The keto-acid which is most prominent in the fruit is pyruvic acid, α-ketoglutaric acid is notable because it is only present in smaller amount, and the keto-acid corresponding to valine (α-keto isovaleric) appeared in unexpected amount. However, the main changes that occur during the ripening process concern pyruvic acid, which decreases sharply as ripening proceeds. This decrease is both on an absolute basis and also relative to the total keto-acid (see Table 3). It will be noted that the alanine content of the fruit also decreases during ripening concomitantly with the decrease in pyruvic acid. In this behaviour fruit from different stalks was consistent. Disparities between the faster ripening Stalk A, in which the α-ketoglutarate increased somewhat with ripening, and the slower Stalk B, in which this acid decreased somewhat, preclude any firm conclusion with respect to this acid.

TABLE 2A

The Amino-Acid Content of the Protein of the Banana Fruit: the Changes that Occur During Ripening

(*Data as N of each amino-acid as per cent. of total protein N*)

	Stalk A	Stalk B					
Days of ripening	0	0	2	4	6	9	12
Amino-acid:							
Cystine	trace	—	—	—	—	—	—
Aspartic acid	9·0	13·7	14·2	11·6	13·5	9·9	9·4
Glutamic acid	10·4	10·7	11·1	11·5	11·0	10·8	10·8
Serine	6·4	4·0	6·2	4·7	5·4	6·0	5·2
Glycine	9·0	7·3	6·3	7·4	7·1	9·7	8·6
Threonine	5·3	3·8	3·8	3·9	4·4	4·9	4·8
α-Alanine	10·0	8·0	7·5	8·2	8·9	10·4	9·6
Histidine	3·8	6·1	6·9	5·4	5·3	4·0	3·3
Lysine	6·3	8·5	7·7	9·2	8·5	8·3	8·6
Arginine	8·0	10·3	7·5	10·0	6·8	5·7	8·9
Proline	3·4	8·3	8·0	6·2	5·0	4·8	4·9
Valine	7·4	5·0	4·9	5·6	6·1	6·7	7·0
Leucine(s)	18·0	12·6	13·0	12·7	13·9	15·7	15·1
Tyrosine	2·8	1·6	1·6	2·2	2·9	3·0	2·8
Hydroxyproline	trace	trace	1·3	1·3	1·3	trace	1·1

TABLE 3

The Keto-acid Content of the Banana (Gros Michel) Fruit: the Changes that Occur During Ripening

(*Data as μg. keto-acid per g. fresh wt.*)

	Stalk A	Stalk B					
Days of ripening	0	0	2	4	6	9	12
Keto-acid:							
α-ketoglutaric	0·45	1·75	—	0·42	1·00	0·64	trace
pyruvic	11·40	14·02	5·64	6·05	3·00	2·15	1·78
β-hydroxypyruvic	0·56	0·79	0·91	0·30	0·72	0·46	0·28
α-ketoisovaleric	2·23	1·64	2·06	trace	2·16	2·40	1·67
succinic semialdehyde	0·48	0·79	trace	0·95	0·90	0·83	0·67

The changes in the amino-acid composition of the banana protein during ripening

From the alcohol-insoluble material, treated as indicated above, it was possible to determine by paper chromatography the amino-acid composition of the bulk protein at each of the stages indicated. The data will be presented as the nitrogen represented by each amino-acid as a percentage of the total protein nitrogen recovered, but also including the total protein—N expressed as microgrammes of nitrogen per g. fresh weight.

The amount of the nitrogen which was originally present in the alcohol-insoluble nitrogen fraction, and which was recovered after hydrolysis as amino-acid by ninhydrin determination, was 80 per cent. of the total for

Stalk B. The protein nitrogen content of the banana pulp, as determined by Kjeldahl determinations, remained relatively constant through the ripening process (see Table 1). Therefore, the only question is whether its amino-acid composition changed appreciably during this period. The test of this is the nitrogen of each amino-acid in the hydrolysate expressed as a percentage of the total nitrogen which was recovered by the ninhydrin method.

These data (Fig. 3) are concordant and show that the nitrogen present as protein in the banana remains approximately constant in amount during

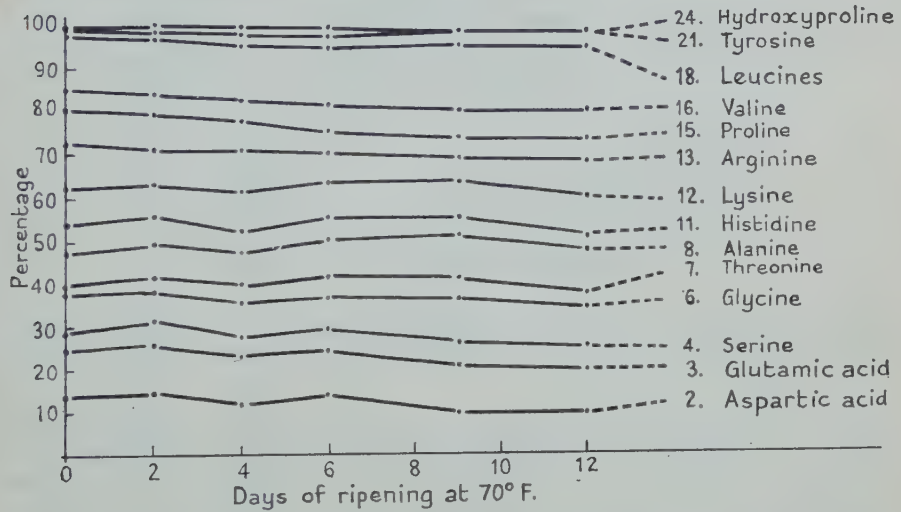

FIG. 3. Alcohol-insoluble nitrogen of the fruit of the banana—changes occurring during ripening. (Nitrogen of each compound as per cent. of nitrogen of hydrolyzed alcohol-insoluble residue determined by ninhydrin.)

the ripening process, and its distribution as between the different amino-acids is also very constant.

It is significant, therefore, that the relative content of histidine in the protein did not tend to increase *pari passu* with its increase in the alcohol-soluble fraction (see Fig. 1). Therefore, the most noticeable change in the soluble-nitrogen fraction, namely the relative increase of histidine, *is quite independent of any changes that occur in the protein.* This being so, the nitrogen which tends to appear as histidine must come from a non-protein source, and this appears to have been the amide fraction (asparagine and glutamine). Therefore, the conversion of amide nitrogen to histidine nitrogen, *without apparent passage into protein*, seems to be a major feature of the banana fruit. The conspicuous change recorded in Table 2 was from glutamine to histidine but in other situations to be discussed later this may vary in detail, especially with respect to the relative roles of asparagine and glutamine. These data are therefore of great interest, because they establish the feasibility of this reciprocal relationship between these two prominent constituents of the soluble nitrogen of the banana fruit.

B. The Soluble Nitrogen Compounds of the Fruit of Different Banana Cultivars and the Changes which Occur During Ripening

The cultivars and locations that describe this range of sampled material are shown in Table 4. All of these samples were obtained during the summer months.

Table 4

Banana Fruit Samples

(Cultivars of *Musa acuminata*)

Cultivar	Stage of ripening*
Lacatan	1, 4, 7
Cavendish	1, 4, 7
Gros Michel (Jamaica) . .	1, 4, 7
Gros Michel (Br. Cameroons)	2, 4, 6

* This is denoted by the colour index on the chart following page 108 of von Loesecke (1950).

The alcohol extracts of the tissue obtained in this way were freed from interfering substances that would otherwise have complicated the paper chromatography, as follows:

In order to prevent decomposition of some of the amino-acids during the process, this procedure was carried out in a cold room at 4° C. Dowex 50×4 was the resin used. The resin was charged by passing 2N. HCl through the column until the washings became acidic. Excess acid was then removed by distilled water.

Each extract was then treated as follows: A 2 ml. aliquot (1 ml. for the Lacatan—Stage 1 extract) of the extract was placed on the column. Cationic compounds (amino-acids) were absorbed by the resin and all other soluble compounds were washed through with distilled water. The amino-acids were eluted, first with 0·15N NH_4OH, then with 2N NH_4OH. These two fractions were taken to dryness under an air stream, combined, and dissolved in 2 ml. of distilled water (1 ml. for the Lacatan—Stage 1 extract). The samples were then analysed by the paper chromatographic method.

By determinations prior to, and after, the purification of these extracts, the percentage recovery of the amino-acids subjected to the treatment can be estimated. For the fourteen amino-acids tested the recovery was never less than 85 per cent.

The data obtained are represented in Table 5, and the data are also represented in the histograms shown in Figs. 4 and 5.

These data may be briefly summarized as follows; and compared with the earlier data on the cultivar Gros Michel from Central America.

In all the samples for which analyses are recorded in Table 5 and Figs. 4 and 5, glutamine also represents a much smaller percentage of the alcohol-soluble nitrogen than in the case of the variety Gros Michel from Central

Table 5

The Amino-acids of the Alcohol-soluble Fraction of the Banana Fruit as Affected by Ripening and Cultivar. Nitrogen in each Compound is Calculated as a Percentage of Soluble Nitrogen Recovered and Determined by Ninhydrin

		Cv. Lacatan			Cv. Cavendish			Cv. Gros Michel					
								Jamaica			Br. Cameroons		
Amino-acids		Stage 1	Stage 4	Stage 7*	Stage 1	Stage 4	Stage 7	Stage 1	Stage 4	Stage 7	Stage 2	Stage 4	Stage 6
Aspartic acid	(2)	3·2	1·0	1·3	3·3	1·4	1·4	4·3	4·5	4·7	4·6	1·3	1·2
Glutamic acid	(3)	1·0	0·8	0·8	1·8	0·3	0·3	1·3	1·2	1·2	0·5	0·3	0·9
Serine	(4)	5·9	6·1	6·9	7·3	5·0	8·1	2·8	4·4	5·0	8·7	7·7	7·7
Glycine	(5)	2·6	3·5	2·7	2·8	1·9	2·5	1·1	1·2	1·5	3·2	2·8	3·6
Threonine	(7)	3·2	3·4	2·9	4·6	2·7	3·2	2·5	2·2	1·6	4·1	3·0	3·8
α-Alanine	(8)	7·7	2·2	2·0	12·3	1·9	1·5	6·9	1·5	1·3	11·3	1·6	2·7
Glutamine	(9)	2·8	1·1	2·4	2·3	1·6	1·3	1·2	1·1	1·4	1·7	1·7	1·9
Histidine	(11)	8·5	17·7	16·2	1·8	12·1	8·2	7·2	11·6	7·8	2·2	7·1	5·3
Lysine	(12)	1·8	5·5	2·2	—	4·6	4·1	2·8	5·0	5·3	0·8	4·4	3·5
Arginine	(13)	11·5	10·9	10·2	8·0	10·1	9·5	11·9	24·8	20·9	4·0	9·3	9·0
Valine	(16)	2·9	3·7	8·9	2·3	3·2	5·9	1·6	2·9	7·3	2·5	4·5	8·3
Leucine(s)	(18)	lost	8·7	12·0	4·6	8·8	13·3	3·2	5·5	9·7	9·1	8·9	16·3
Phenylalanine	(19)	lost	1·3	trace	trace	—	—	2·1	trace	—	trace	trace	trace
Tyrosine	(21)	2·3	3·1	2·2	2·5	1·2	1·1	1·1	0·3	0·8	2·1	2·0	—
γ-Aminobutyric acid	(23)	7·7	3·4	6·5	11·1	3·9	4·3	5·1	4·6	4·6	9·2	3·2	5·4
Asparagine	(6)	32·6	23·9	20·6	32·3	38·1	32·9	43·8	26·0	25·1	30·7	37·9	23·9
Proline	(15)	6·5	3·7	2·5	2·9	3·0	2·4	1·8	3·3	1·8	5·4	4·5	6·6
Methionine sulfoxide	(14)	—	some	trace	—	trace	—	trace	trace	trace	trace	trace	trace
Phenyl-alanine	(22)	—	trace	trace	—	trace	—	—	—	some	trace	trace	—
Hydroxyproline	(23)	—	trace	trace	trace	trace	trace	trace	trace	some	—	—	trace
Pipecolic acid	(26)	—	some	some	some	some	some	some	some	some	some	some	some
Tryptophan	(20)	—	—	—	—	—	?	—	?	?	—	—	?

* Sample contaminated.

America, ripened at Cornell in the autumn (see Fig. 1). However, where glutamine was much less conspicuous in the soluble nitrogen, asparagine remained the most conspicuous component, and arginine was as prominent

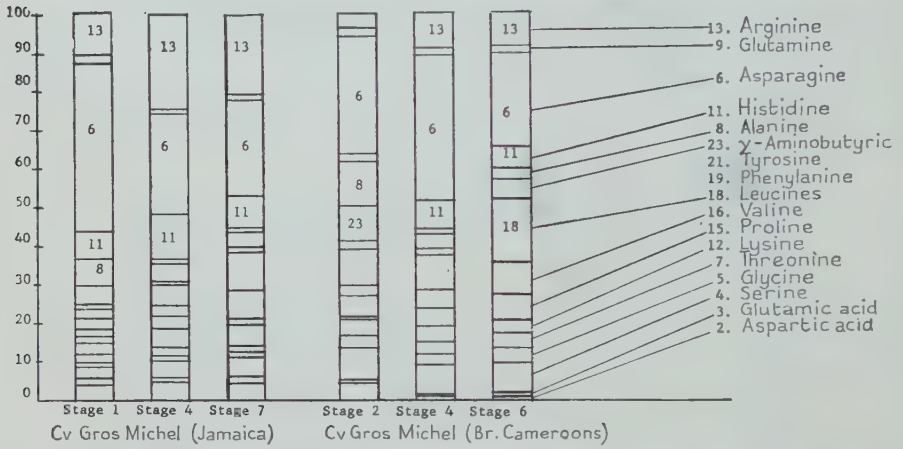

FIG. 4. Alcohol-soluble nitrogen of the fruit of banana—changes during ripening. (Nitrogen of each compound as per cent. of soluble nitrogen determined by ninhydrin.) (These histograms relate to Table 5.)

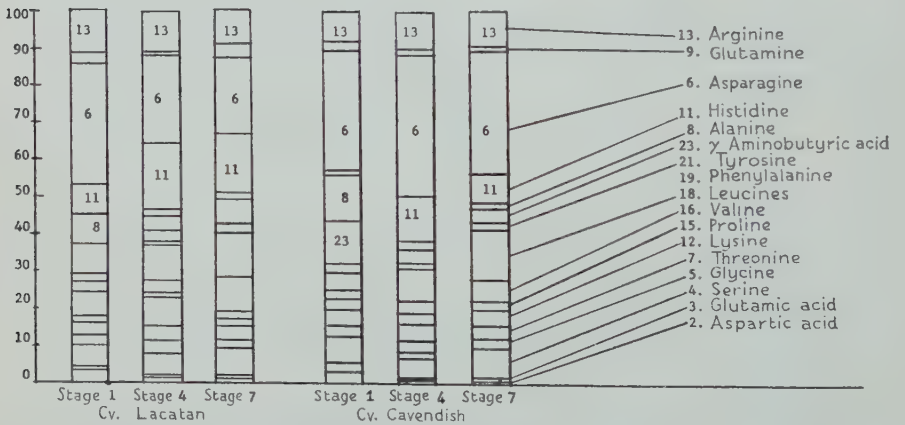

FIG. 5. Alcohol-soluble nitrogen of the fruit of banana—changes during ripening. (Nitrogen of each compound as per cent. of soluble nitrogen determined by ninhydrin.) (These histograms relate to Table 5.)

as in the earlier samples of Gros Michel fruit (see Fig. 2). It will be observed that an outstanding characteristic of the banana fruit in this series of observations, as well as fruit shown in Fig. 2, is that the alcohol-soluble nitrogen was more widely distributed amongst a variety of nitrogen compounds than the few (asparagine, glutamine, histidine) in which it was concentrated in the material of Fig. 1. Reference to Figs. 4 and 5 will show that from 40 to 60 per cent. of the alcohol-soluble nitrogen was here distributed between

nitrogen compounds other than asparagine, glutamine, histidine, and arginine, and some of these compounds became relatively prominent constituents. This can be seen for such substances as serine, proline, valine, and leucine of the ripened Gros Michel fruit which was obtained from the Cameroons; and this can be contrasted with the data from the same variety from Central America (Fig. 1), in which none of these substances acquired the same degree of prominence in the soluble nitrogen, although serine and the leucines showed some tendency to do so.

Another prominent difference between the Gros Michel fruit of Figs. 1 and 2 and the fruits of Figs. 4 and 5 is the fact that the former contained substantial amounts of pipecolic acids whereas, in the latter, it was not observed in amounts that could be readily determined.

While histidine usually appeared in this wider range of banana pulp samples, and again showed some tendency to increase during ripening (e.g. Lacatan, Cavendish, and Gros Michel from Br. Cameroons), this effect was neither as marked, nor as consistent, as in the Gros Michel fruit which furnished the data of Table 2 and Fig. 1. In all these materials asparagine, and not glutamine, was the prominent amide.

It will be evident that the data recorded in Figs. 4 and 5 show a somewhat surprising degree of uniformity, despite disparities in both the cultivars and the place of origin of the fruit; and it will also be observed that this group of data as a whole contrast markedly with the data of Fig. 1 in ways which have been mentioned above. It is clear, therefore, that the prominent conversion of amide (glutamine) nitrogen to histidine, which was seen to occur in a particular sample of Gros Michel fruit from Central America, may not be an invariable property of the ripening of all banana fruit. Indeed, the basic amino-acid arginine also shows some tendency to be more conspicuous in the pulp of ripened Gros Michel fruit which, because of origin, had different characteristics which are best indicated by the low initial glutamine content. Thus, in some degree, the increase of arginine in the tissue on ripening here tended to supplement the effects earlier described for histidine.

The main conclusion, however, must be that the changes in the soluble-nitrogen compounds of ripening banana fruit can be affected, within broad limits, by the treatment to which the fruit is subjected, either during shipment or during its growth. The variables which determine this must certainly override the cultivar differences alone, for the variations between *different samples of Gros Michel fruit are actually larger than those between samples taken from different cultivars.*

It has to be recognized that the rather complete data of Table 2 and Figs. 1 and 2 for the ripening of Gros Michel fruit from Central America depends necessarily on the limited range of samples that have been studied to date. Since these represent the only data of their kind, their publication is justified because it draws attention to this hitherto unrecognized behaviour of the fruit. In doing so, however, it is recognized that disparities in the fertilizer practice in the areas of cultivation, and the different seasons of

the year in which the fruit is harvested may have a bearing on the biochemical responses of the fruit.

It now seems clear that the alcohol-soluble nitrogen fraction of the banana fruit, at the stage of maturity that is reached before shipment, represents a fluid pool of metabolites which may not only differ in initial composition but may also change in a variety of ways during the processes of storage and ripening. The most prominent initial constituents seem to be the amides asparagine and glutamine, the basic amino-acid histidine, and arginine. One can recognize certain trends that are much more conspicuous in what may be called '*high-glutamine*' *fruit*, and these are notably a greater tendency for histidine to accumulate at the expense of amide nitrogen, mainly glutamine, as ripening proceeds and a greater emphasis upon the presence of pipecolic acid. In this connexion it is of interest to note that Neidle and Waelsch (1956) have shown that *Escherichia coli* will bring about a high incorporation of N^{15} labelled amide-N of glutamine into N-1 of the imidazole ring of histidine and in the presence of large quantities of asparagine or ammonia. What may be called '*low-glutamine*' *fruit* shows by contrast a greater tendency to emphasize asparagine and arginine in relation to histidine, and a much greater tendency to distribute the nitrogen in the soluble pool amongst a variety of the common amino-acids, notably serine, valine, leucine, and proline. After the behaviour of the more major components of the soluble-nitrogen fraction are considered, some further trends in the behaviour of the more minor constituents may be noted. For example, the content of alanine, aspartic acid, and γ-aminobutyric acid frequently tends to fall during ripening, while the content of valine and the leucines tends reciprocally to increase.

At this point, therefore, it seems more important to recognize the range and variety of changes to which the soluble nitrogen compounds of the banana fruit are subject than to attempt to explain them. It is apparent that the biochemical implications of the alcohol-soluble nitrogen fraction are much greater when scrutinized in terms of the individual substances that it contains than when attention is merely devoted to the apparent constancy of the soluble pool of nitrogen as a whole. If guiding principles are to be sought at this point, it seems profitable to do so along the lines that the factors which make for prominence of glutamine and histidine on the one hand, or of asparagine and arginine on the other, may reflect deep-seated biochemical differences in the fruit, whether these are largely induced by nutrition (e.g. as by sources of nitrogen such as urea), climatic conditions (e.g. as by the season during which the fruit matures), or by conditions which obtain during storage and shipment prior to the post-climacteric ripening. The data to confirm all these possibilities, probable as they are, are not yet available. Inasmuch, however, as the examination of the alcohol-soluble fraction in the fruit by modern chromatographic procedures enables a large area of metabolism to be scanned, it furnishes a rapid means of appreciating the metabolic pathways which are affected by a variety of experimental and environmental variables in ways which might otherwise be overlooked.

The amino-acid composition of the total protein of the fruit as affected by cultivar stage of ripening, and origin

The earlier references which have been made to the relative amino-acid composition of the alcohol-insoluble nitrogen of the fruit have stressed that this fraction is much more stable in its composition during ripening than is the alcohol-soluble fraction. However, by combining into one table the results now available for various cultivars (e.g. Gros Michel, Cavendish, and Lacatan), and from various localities (e.g. Central America, Jamaica, and

TABLE 6

Amino-acid Analysis of the Hydrolysates of the Insoluble Residues of Banana Fruit as Affected by Ripening and Cultivar. Nitrogen in each Compound is Calculated as a Percentage of Soluble Nitrogen Recovered as Determined by Ninhydrin

		Cv. Lacatan			Cv. Cavendish	Cv. Gros Michel				Cv. Gros Michel Central America Stalk B	
						Jamaica		B. Cameroons		Days of Ripening	
										0	12
Amino-acids		Stage 1	Stage 4	Stage 7	Stage 1	Stage 1	Stage 4	Stage 2	Stage 4	Stage 1	Stage 6–7
Aspartic acid	(2)	8·4	9·6	6·4	6·5	15·1	12·4	8·7	9·7	13·7	9·4
Glutamic acid	(3)	6·2	5·7	5·0	7·1	6·1	4·2	7·3	8·3	10·7	10·8
Serine	(4)	2·5	3·3	3·2	5·1	2·9	5·2	2·7	4·2	4·0	5·2
Glycine	(5)	2·5	3·9	5·8		2·2	3·4	1·8	3·6	7·3	8·6
Threonine	(7)	3·7	3·8	4·0	2·1	2·2	3·9	2·1	4·5	3·8	4·8
Alanine	(8)	4·8	8·3	7·6	4·8	3·4	6·9	6·2	8·3	8·0	9·6
Histidine	(11)	2·6	3·9	3·1	2·1	10·1	6·0	2·2	3·2	6·1	3·3
Lysine	(12)	7·0	5·5	8·1	7·5	7·5	7·4	6·9	7·0	8·5	8·6
Arginine	(13)	20·3	23·0	20·0	24·4	29·9	25·5	25·0	20·8	10·3	8·9
Valine	(16)	5·5	6·2	6·3	6·8	5·8	4·7	7·1	6·4	5·0	7·0
Leucine(s)	(18)	15·5	14·7	13·6	17·0	9·2	10·3	16·8	13·6	12·6	15·1
Phenylalanine	(19)	4·1	2·6	5·0	3·6	2·4	2·8	5·4	3·9	—	—
Tyrosine	(21)	1·2	2·1	4·6	1·9	1·1	0·4	2·0	4·0	1·6	2·8
Proline	(15)	12·6	6·5	7·2	11·1	1·6	5·3	5·8	2·6	8·3	4·9
Methionine sulfoxide	(14)	trace	trace	trace	—	—	—	trace	—	—	—
Hydroxy-proline	(24)	3·1	1·0	trace	trace	0·6	1·6	trace	trace	—	—

British Cameroons) for the cultivar Gros Michel, one can begin to see that the amino-acid composition of the total alcohol-insoluble fraction is not constant but is subject to certain discernible trends.

The data of Table 6 have been assembled by substantially the same methods throughout; that is, the alcohol-extracted tissue was subjected to acid hydrolysis, re-extracted for the amino-acids in the hydrolysate, and these were then determined by the same procedures of quantitative chromatography. From the amounts of amino-acids so determined in milligrams their equivalent in nitrogen was calculated, and the relative composition in the table is expressed on the basis of the percentage of nitrogen present in each amino-acid, as a percentage of that recovered in the hydrolysate by the ninhydrin method. The data for the variety Gros Michel from Central America (Stalk B, at two stages of ripening) are placed in the table for

comparison with the rest of the data; and it will be noted that these data relate to the tissue which showed the strikingly different composition in the soluble nitrogen and the evidence of glutamine–histidine conversion on ripening (see Fig. 1 and Table 2).

The first point is that the tissue from Central America (last two columns of Table 6) which has been referred to as 'high-glutamine' fruit and in which the content of histidine in the soluble nitrogen was accentuated, and that of arginine minimized, proves to have a composition in its protein which is distinctively different from all the rest of the samples recorded in the table. It is clear that the protein content of this fruit had a lower arginine content, some 10 per cent. of the protein nitrogen being represented by arginine compared with 20 to 25 per cent. in all the other samples; and it also had a somewhat higher content of the dicarboxylic acids (some 20 to 24 per cent. of the nitrogen of the protein present is aspartic and glutamic acid, compared with a somewhat lower figure in the other materials). It is possible, therefore, to recognize characteristics in the alcohol-insoluble nitrogen fraction which rendered this 'high-amide', 'high-histidine', type of fruit somewhat distinctive in its protein nitrogen composition also. By contrast, all the other diverse materials examined, irrespective of cultivar and place of origin, show a rather remarkable uniformity in the analysis of the protein.

When scanning the entire results shown in the table, it now becomes possible to see certain trends that suggest that the protein fraction does change somewhat in its composition as the ripening process proceeds. The points that are relevant here are as follows:

(*a*) The combined contribution of serine, glycine, and threonine to the total protein nitrogen seems consistently to increase in the ripened fruit. This is shown by the change from 8·7 to 13 per cent. in the variety Lacatan; from 7·3 to 12·5 per cent. in the Gros Michel from Jamaica; from 6·6 to 12·3 per cent. in the Gros Michel from the Cameroons; and from 15·1 to 18·2 per cent. in the Gros Michel material of Stalk B from Central America. By contrast the total contribution of the dicarboxylic acids to the protein nitrogen tends to decrease, as shown by the change from 14·6 to 11·4 per cent. for the variety Lacatan; from 21 to 16 per cent. in the variety Gros Michel from Jamaica; and from 24·4 to 20·2 per cent. for the Gros Michel material of Stalk B from Central America. Similarly there is a consistent tendency for the contribution of alanine to the protein nitrogen to increase. This is shown by the change from 4·8 to 7·6 per cent. in the case of Lacatan; from 3·4 to 6·9 per cent. for Gros Michel from Jamaica; from 6·2 to 8·3 per cent. for the Gros Michel from the Cameroons; and from 8 to 9·6 per cent. in the case of the special material from Stalk B in Central America. With the exception of one case—that is, the variety Gros Michel from Jamaica—the contribution of proline to the total protein nitrogen consistently decreased during the ripening. Amongst amino-acids which seem to remain rather stable in their contribution to the total protein nitrogen are the amino-acids lysine, valine, and leucine.

These figures, therefore, seem to indicate that the protein nitrogen, though much more stable in its amino-acid composition than the soluble nitrogen nevertheless does undergo some changes during the ripening process; and it also indicates that the differences between tissue which showed high glutamine and histidine content on the one hand, and high asparagine and low histidine but higher arginine content on the other, also tend to affect the composition of the protein fraction in ways which can only be noted though not, at this stage, explained.

C. Seasonal Effects on the Nitrogenous Composition of the Banana Fruit

The foregoing discussion has emphasized that there are variables at work to determine the distribution of nitrogen amongst a variety of soluble compounds in the fruit and, though to a much less extent, to determine the distribution of nitrogen amongst the amino-acids of the protein. While stage of maturity and ripening obviously have effects upon these aspects of the metabolism of the fruit, it is clear that there are other variables at work which over-ride the effects of the cultivars. To identify these factors fully is clearly beyond our present knowledge, but there are now available enough data to indicate the importance of the time of year at which the fruit develops and matures.

Broadly speaking, one may distinguish for Central America fruit which grows and matures largely in the warmer summer months (i.e. April to August), and fruit which develops by contrast through the cooler winter months (i.e. October to February). The general temperature conditions that prevail in these two periods affect the rate at which the fruit develops (approximately 90 days from emergence in the warmer to 120 days in the cooler). The material which furnished the data of Table 2 and Fig. 1, and in which amide and histidine played such a conspicuous role in the soluble-nitrogen fraction, was material received in the U.S.A. in the late autumn, and was therefore material which was intermediate between these two extremes. Therefore, it is of interest to compare material collected in Central America at other seasons of the year, sampling these at a stage of development comparable to that at which fruit is normally harvested for shipment to the U.S.A. Although the data available are not as extensive as might be desired, nor have they been assembled systematically throughout the year, they are at least indicative of the kind of differences that may be attributed to the season of the year at which the Gros Michel fruit develops and reaches the sampling stage.

To test the seasonal effects on the composition of the unripened Gros Michel fruit two further series of samples were collected in Honduras in July 1956, and in December of the same year and from the same locality, when the fruit had reached the same stage of development. These samples were treated in the manner which has been reported and were analysed for the soluble-nitrogen compounds. Since the bulk of the soluble nitrogen is

represented by the two amides asparagine and glutamine and the basic amino-acid histidine, it will be convenient to compare the seasonal effects by reference to these substances only.

Table 7 therefore shows the changes which occur in the proportions of glutamine, asparagine, and histidine, expressing these as the percentage of the nitrogen which they contribute to the total soluble nitrogen as determined. The amount of nitrogen represented by the total amide plus histidine is remarkably constant at 69 to 75 per cent. It is also evident that the time of the year when the fruit developed and was harvested determines the relative

TABLE 7

Seasonal Effects in the Proportion of Asparagine, Glutamine, and Histidine Present in the Alcohol-Soluble Nitrogen of Gros Michel Fruit at Harvest

(*N of each amino-acid as per cent. of total soluble nitrogen determined*)

Condition	Summer-grown fruit	Intermediate		Winter-grown fruit
Time of harvest	July	October		December
Days from emergence	95	—		115
Asparagine	35·1	32·1	27·4	26·8
Glutamine	25·6	27·7	22·5	7·4
Total amides	60·7	59·8	49·9	34·2
Histidine	8·2	15·4	21·4	35·0
Amides + histidine	68·9	75·2	71·3	69·2

importance of amide and histidine nitrogen in the soluble fraction, there being a progressive decrease in amide nitrogen from July to December and concurrently a progressive increase of histidine nitrogen.

Also from Table 7 it can be seen that the longer the time required for the fruit to develop, the more marked is the shift in the soluble nitrogen toward histidine, and away from the amides, especially from glutamine. It will be observed that this is exactly the trend that was still further accentuated during the ripening process as this was recorded in Table 2 and Fig. 1. On the other hand, fruit ripened during the winter months and containing a high histidine content at harvest time maintained histidine at a high level and glutamine at a low level during the ripening period (see Fig. 2).

D. Effects due to the Stage of Development of Banana Fruit

The growth curve of the developing fruit

The first objective in this section was to appraise the way in which the nitrogenous compounds of the banana fruit change during the stages of initiation, development, and maturation to the stage at which it is normally harvested. In the interpretation of the developmental series the following facts should be kept in mind. The vegetative shoot apex undergoes floral initiation some weeks before the inflorescence emerges from the pseudostem, and the date of emergence is a convenient reference point along the growth curve of the individual banana fruit. At approximately 30 days prior to

emergence the inflorescence can be readily recognized and detected, but it is still small, only a few centimetres in overall length. Later, as the inflorescence moves upward in the pseudostem, more mature stages can be obtained; and the size of the individual fruit ovaries may be measured and related to their age. At the time when the inflorescence emerges, however, the individual fruits are still only a small fraction of their full size. The method adopted was to sample fruit material at various points along this sequence of development. Samples were taken at four stages from the time of emergence when

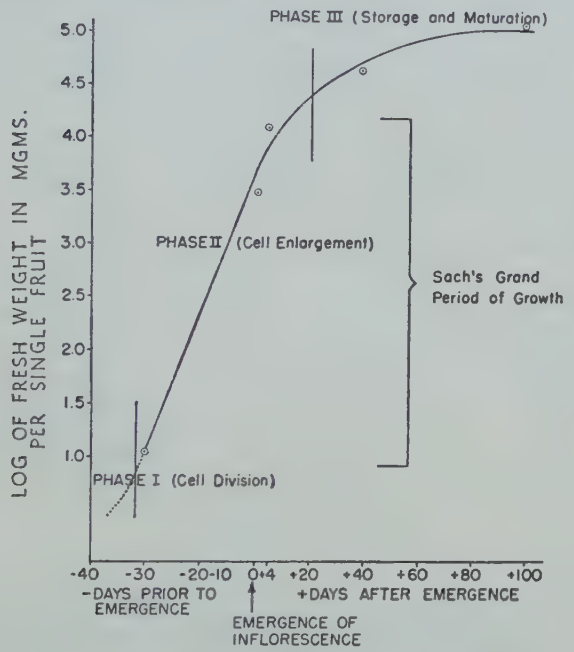

Fig. 6. Growth curve of single Gros Michel fruit.

the individual fruit fingers weighed 2·6 g. up to the time of normal harvest when they weighed 178 g. In the youngest material available for study, namely approximately 30 days prior to emergence, the whole inflorescence was treated as a single sample. The general form of the growth curve for the individual banana ovaries which subsequently develop parthenocarpically into the fruit is illustrated at Fig. 6. The figure relates the logarithm of the fresh weight in milligrams for a single fruit to the days prior and subsequent to emergence of the inflorescence from the pseudostem.

The general sigmoid form of the growth curve shown in Fig. 6 is typical for any organ of this kind, and it can be approximately divided into three main phases.

In the first phase, the fruit as an organ is being initiated and one may suppose that cell division is here prominent; in the second protracted phase of rapid elongation, the growth is proceeding exponentially with time and the

logarithmic growth curve is approximately linear. This phase lasts for approximately 60 days and is conspicuous by the great enlargement of the organ, corresponding to Sachs's 'grand period of growth'. Thereafter the organ, which has now almost reached its full size, can be regarded as in the phase in which it is acquiring storage products and is approaching maturation.

The total soluble nitrogen of the fruit during development: seasonal effects

The first objective, then, is to determine how this sequence of developmental stages is reflected in the nitrogenous composition of the individual fruit.

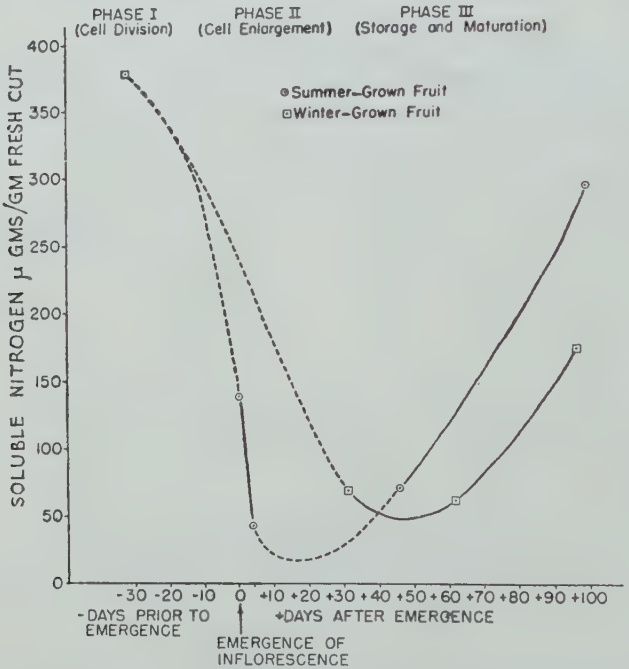

FIG. 7. Change in total soluble nitrogen of Gros Michel fruit during development.

This will be done first with reference to the total soluble nitrogen (microgrammes per gramme fresh weight) as determined for fruit of specified size. Knowing that fruit develops in the winter and summer periods at a different rate, and that this is reflected in its composition as already indicated, this type of analysis has been carried out at two different seasons of the year. The data are shown on Fig. 7 and relate to 'summer-grown' and 'winter-grown' fruit respectively.

The general interpretation of Fig. 7 seems clear. During the rapid exponential growth of the fruit, its initially high soluble-nitrogen content is depleted; this is an indication of the more rapid utilization of nitrogen in synthesis than its replacement by importation into the growing fruit. The

soluble-nitrogen content of the fruit thus reaches its *lowest level a few weeks after the inflorescence emerges*. Thereafter, and increasingly as the fruit reaches its mature size, the soluble-nitrogen content is regained as importation of nitrogenous substances occurs in quantity greater than is being used for protein synthesis. However, the relative composition of the soluble nitrogen which is recovered in the later phase of maturation and storage is different from the composition of the nitrogen compounds which are present in the earlier nitrogen-rich immature phase. The general trend of the soluble nitrogen with time is similar in the winter-grown and the summer-grown fruit but, as the summer-grown fruit develops more rapidly, the fluctuation in time is accentuated, reaching its lowest level earlier in the developmental period than the winter fruit, which develops more slowly. Since the winter-grown fruit developed more slowly, it reached by 93 days after emergence a relatively less mature condition, and in fact it did not reach the same stage of maturity till some 115 days from emergence. Through this later period, however, the fruit did not continue to increase in soluble nitrogen in the manner shown for the summer-grown fruit and, in point of fact, an actual decrease occurred in the pulp, though not in the peel. The trends of nitrogen content with development for summer- and winter-grown fruit up to the stages shown in the figure are in general confirmatory, and the pattern of behaviour seems clear, but since the causes of the decline in soluble nitrogen of the pulp in the later stages of the winter-grown fruit are unknown, this fact is noted but left for further explanation.

The composition of the alchohol-soluble nitrogen of the fruit during development at different seasons

Reference to the tables will readily show that there is a very drastic change in the *relative* composition of the soluble nitrogen, as well as in its total amount, through the developmental sequence. The nitrogenous composition of the reproductive tissue in the very young inflorescence is quite different from the composition of the fruit previously reported at harvest. It has been noted earlier that from harvest onwards a large part (of the order of 70 per cent.) of the total soluble nitrogen in the fruit is distributed between the amides and histidine. The very young inflorescence, however, is conspicuously poor in all these substances; and indeed the total amount of nitrogen represented by these three substances represented only about 5 per cent. of the total amount determined. At this stage the bulk of the soluble nitrogen, amounting to approximately 63 per cent. of the total, was represented by the amino-acids alanine, arginine, proline, and γ-aminobutyric acid and, including the dicarboxylic acids, 80 per cent. of the soluble nitrogen would have been accounted for thereby. By contrast, as already indicated, at the stage of harvest the pulp achieves another nitrogen-rich condition, but the nitrogen now consists predominantly of the amides (asparagine and glutamine) and histidine.

The main transitions that occur through this developmental sequence in

banana pulp are as follows: (*a*) While the total amount of alanine contained in the tissue is highest at the very youngest stage, the percentage of the total soluble nitrogen which is represented by this amino-acid remains relatively constant till about the time of emergence, when it tends to fall to a much lower level. A similar trend apparently also applied to γ-aminobutyric acid. (*b*) The dicarboxylic acids aspartic acid and glutamic acid consistently decline in their importance in the soluble nitrogen fraction as development proceeds;

TABLE 8

Effect of Stage of Development of 'Summer-grown' Banana Fruit (Gros Michel) on the Alcohol-soluble Ninhydrin Reactive Substances

(*N of each amino-acid expressed as per cent. of the total soluble nitrogen*)

Amino-acid		Days after emergence of fruit			
		0	4	45	95*
Aspartic acid . .	(2)	7·1	9·1	10·7	5·8
Glutamic acid . .	(3)	9·7	14·4	6·6	2·9
Serine, Glycine .	(4, 5)	4·2	1·1	3·2	2·0
Asparagine . . .	(6)	6·6	13·1	18·8	35·1
Threonine . . .	(7)	2·6	3·3	2·2	1·8
Alanine . . .	(8)	14·0	9·9	6·8	4·8
Glutamine . . .	(9)	30·3	5·8	31·2	25·6
Histidine . .	(11)	—	—	—	8·2
Lysine . .	(12)	1·7	—	—	1·2
Arginine . .	(13)	trace	some	—	trace
Proline . .	(15)	2·0	13·8	2·5	0·9
Valine . .	(16)	4·1	6·2	2·2	0·9
Leucines . .	(18)	2·8	5·8	1·9	1·2
Tyrosine . .	(21)	2·5	3·9	1·9	0·7
β-Alanine . .	(22)	trace	trace	trace	trace
γ-Aminobutyric acid	(23)	11·8	9·9	10·8	8·4
Pipecolic acid .	(26)	0·8	2·4	some	trace
Total micrograms of soluble N per g. of fresh wt.		134·9	45·2	69·4	292·4

* Stage of harvest.

that is from 16 per cent. in the youngest material analysed down to approximately 4 per cent. in the most mature fruit. (*c*) Serine and glycine as a composite fraction represent about 9 per cent. of the total soluble nitrogen of the most immature inflorescence, whereas at harvest they represent only about 2 per cent. of the soluble nitrogen in the fruit at harvest.

Therefore, the nitrogen of the amino-acids as a percentage of the total soluble nitrogen tends to decrease during development for almost all the substances present; that is, aspartic acid, glutamic acid, serine, lysine, threonine, alanine, arginine, proline, valine, tyrosine, and γ-aminobutyric acid. In conspicuous contrast, the three substances asparagine, glutamine, and histidine increase markedly as a percentage of the total soluble nitrogen during the time of development so that by 61 days after the fruit has emerged these substances represent about 70 per cent. of the total soluble nitrogen

determined. The data from which the above generalizations have been drawn are to be found in Tables 8 and 9.

It will be noted that a developmental trend is presented for fruit pulp which matured at different seasons of the year (see Tables 8 and 9). From what has already been presented differences in the soluble-nitrogen composition of the fruit are to be expected and have been shown for the composition at the time of harvest. It is evident, therefore, that one would expect

TABLE 9

Effect of Stage of Development of 'Winter-grown' Banana Fruit (Gros Michel) on the Alcohol-soluble Ninhydrin Reactive Substances

(*N of each amino-acid expressed as per cent. of the total soluble nitrogen*)

Amino-acid		Young inflorescence minus 30	Days after emergence of fruit: Pulp 31	Pulp 61	Pulp 93	Pulp 115*	Peel 31	Peel 61	Peel 93	Peel 115*
Aspartic acid	(2)	8·1	10·0	4·0	5·1	2·3	8·2	1·8	3·5	8·8
Glutamic acid	(3)	8·8	6·5	3·7	2·8	1·6	3·4	3·6	7·0	5·9
Serine	(4)	7·0	7·1	2·9	2·3	1·5	6·8	1·8	4·7	3·7
Glycine	(5)	2·1	0·6	0·7	0·9	0·7	1·4	trace	1·2	1·7
Asparagine	(6)	5·4	36·6	51·8	54·4	26·8	10·9	trace	trace	25·4
Threonine	(7)	1·8	3·2	0·5	2·0	0·8	0·7	—	0·5	3·9
α-Alanine	(8)	13·6	2·1	3·2	5·1	4·8	12·9	10·7	14·0	8·6
Glutamine	(9)	trace	—	21·0	9·5	7·4	4·8	3·6	3·5	15·9
Histidine	(11)	—	7·3	—	2·4	35·0	—	—	trace	—
Lysine	(12)	—	3·6	0·8	2·0	3·3	—	—	—	3·2
Arginine	(13)	12·5	8·6	trace	3·7	5·3	4·1	—	trace	2·9
Methionine sulfoxide	(14)	—	—	—	—	—	—	1·8	—	—
Proline	(15)	21·2	trace	—	trace	trace	—	8·9	—	trace
Valine	(16)	2·0	0·6	trace	0·4	1·6	—	—	0·6	1·5
Leucines	(18)	—	—	—	trace	0·4	—	—	—	1·0
Phenylalanine	(19)	—	—	—	—	trace	trace	—	—	trace
Tyrosine	(21)	1·7	1·4	—	trace	trace	—	—	—	—
γ-Aminobutyric acid	(23)	15·9	12·5	11·4	9·5	8·6	46·9	67·9	65·1	17·6
Pipecolic acid	(26)	—	some	some	some	some	some	some	some	some
Total micrograms of soluble N per g. of fresh wt.		372·4	66·2	59·5	173·6	103·4	14·7	5·6	8·6	40·9

* Stage of harvest.

the changing composition of the fruit with development to vary with the season of the year. This can be illustrated with respect to asparagine and to histidine. The winter-grown fruit (see Table 9), clearly becomes asparagine-rich earlier in time during development than does the summer-grown fruit. For example, by 31 days after emergence the asparagine in the winter-grown fruit represented 36 per cent. of the total soluble nitrogen, and by 93 days had reached the very high figure of 54 per cent., whereas in the summer-grown fruit (see Table 8) even after 45 days from emergence it was still at a level as low as 19 per cent. Similarly histidine was observed as early as 31 days from emergence in the winter-grown fruit, and reached the very high value of 35 per cent. at harvest; whereas in the summer-grown fruit the histidine was not observed prior to harvest and at harvest it had only reached a value of 8 per cent.

The peel was removed from the fruits that developed during the winter

season and was separately analysed. The data are presented in Table 9 along with the analysis of the pulp at the same sampling periods. As in the case of the pulp, the soluble-nitrogen content of the peel first declines during development to a low level and then increases as maturity is approached. There are outstanding differences in the nitrogenous composition of the peel and the pulp, especially because the substance histidine, which is so prominent in the soluble nitrogen of the pulp, may be absent from the peel.[1] Although the amount of soluble nitrogen in the peel is quite low compared to that of the fruit (6 to 41 μg. per gm. peel compared to 60 to 174 μg. N per gm. pulp), certain amino-acids are prominent in this fraction. Gamma-aminobutyric acid, which is the most prominent substance in the peel, accounted for as much as 68 per cent. of the total soluble nitrogen in certain cases, with alanine being the next most prominent amino-acid. The two amides asparagine and glutamine increase notably in the peel as the development proceeds and naturally γ-aminobutyric acid is, consequentially, reduced in importance.

E. The Distribution of Nitrogenous Compounds Within the Fleshy Fruit

The parthenocarpic fruit of the banana comprises a complex pericarp which surrounds the placental region, on which are borne the vestigial ovules. The outer pericarp rind has distinctive amino-acid composition in its alcohol-soluble fraction (see Table 10). The sampling technique which has been adopted in this work has treated the entire fleshy pulp as a unit, but this includes the inner fleshy pericarp (endocarp) and the region which includes the placental. The striking changes which occur in the nitrogen compounds of the fruit during its development and during the ripening process aroused the suspicion that the morphologically different regions of the fleshy pulp may *not* be physiologically or biochemically comparable.

Ripe fruit (Stage 8 on the standard scale) was obtained, the rind was removed and discarded. From transverse disks of the pulp the central placental region was removed with a cork borer in such a way that the outer region (fleshy pericarp or endocarp) was slightly contaminated with the inner region, but not the reverse. The alcohol extracts of these two regions were then analysed after preliminary treatment on resins to obtain the amino compounds free of the interfering substances which otherwise cause the chromatograms to streak. The very striking results are given in Table 10.

The essential results are clear. In the ripened fruit the total soluble nitrogen is very much greater per unit weight of tissue in the inner or placental region (almost *four times the amount* in the inner, fleshy, pericarp). Whereas *all* the ninhydrin-reactive compounds determined were present at higher levels in the placental region than in the endocarp, the amino-acid composition of the two regions was quite different. Virtually *all* the amide of the ripe fruit was in the inner region, though in *both* histidine was high, as

[1] Other data, from samples harvested in Jamaica, suggest that there are still seasonal and other effects to be ascertained.

now repeatedly observed for Gros Michel from Honduras. Although the absolute amount of histidine per unit weight of tissue was greater in the inner region, it actually represented a higher percentage (37%) of the nitrogen of the outer region than of the inner (26%). Whereas approximately 30-per cent. of the soluble nitrogen of the inner region was accounted for by the amides (asparagine 16·7%; glutamine 12·7%), these substances together only accounted for less than 2 per cent. of the nitrogen in the fleshy part of the

TABLE 10

Alcohol-soluble Nitrogen of the Carpellary Region of the Banana Fruit and the Surrounding Fleshy Pericarp (Endocarp) at Stage 8 of Gros Michel

Nitrogen (N) in each compound is calculated as: (1) a percentage of soluble nitrogen recovered and determined by ninhydrin; and (2) micrograms of nitrogen per g. fresh wt.

	μg. N of each compound per g. F. W. of tissue		N of each compound as % of Sol. N	
	External	Internal	External	Internal
Aspartic acid	1·1	15·1	0·9	3·3
Glutamic acid	0·8	10·5	0·6	2·3
Serine	10·1	28·0	8·3	6·1
Glycine	3·0	5·0	2·4	1·1
Asparagine	trace	76·0	trace	16·7
Threonine	2·7	5·2	2·2	1·1
Alanine	1·8	7·4	1·4	1·6
Glutamine	1·9	57·8	1·5	12·7
Histidine	44·9	118·0	36·9	25·9
Lysine	1·9	5·0	1·4	1·1
Arginine	5·6	20·6	4·6	4·5
Methionine sulfoxide	some	0·9	some	0·2
Valine	12·0	24·2	9·8	5·3
Leucine(s)	12·6	30·7	10·3	6·7
Phenyl alanine	1·3	2·6	1·0	0·5
Tyrosine	3·3	8·4	2·7	1·8
β-Alanine	0·9	0·9	0·7	0·2
γ-Aminobutyric acid	5·2	13·9	4·2	3·0
Pipecolic acid	12·4	24·0	10·2	5·2
Proline	trace	trace	trace	trace
Total	121·5	454·2		

pericarp, and even this is open to the suggestion that it was due to contamination of the sample with the inner region. Leucine (10·3%), valine (9·8%), serine (8·3%), arginine (4·6%), and γ-aminobutyric acid (4·2%), in that order, contributed 37·2 per cent. of the soluble nitrogen of the outer region and, surprisingly, pipecolic acid accounted for 10 per cent. Although γ-aminobutyric acid was the prominent amino-acid of the rind (outer pericarp), it was not so in the fleshy or inner (pericarp).

Clearly, therefore, the different morphological regions of the fleshy fruit have quite different biochemical composition, and logically their separate behaviour throughout the whole process of development and ripening should now be traced. This will require further work, but meanwhile the following speculation may be permissible.

The contents of the loculus probably reflect the rich deposition of soluble-nitrogen compounds which otherwise would have been used in the development of the ovule. (The unlikely possibility that this arose from protein breakdown during ripening can be discounted, because the protein content of the fruit as a whole is so constant.) The fact that *both* amide and histidine remained at such high levels within the loculus probably precludes much evidence of the amide (glutamine) to histidine change, which has earlier been recognized in the fruit. On the other hand, the virtual absence of glutamine from the outer fleshy pericarp and the high relative concentration of histidine suggest that the glutamine–histidine conversion on ripening may occur primarily in this region.

Summary, Discussion, and Conclusions

1. Against the background of knowledge of the composition of the banana fruit, which was obtained by the use of chromatographic methods (Part I), certain changes that occur during ripening of the fruit of the cultivar Gros Michel and in certain other cultivars of *Musa acuminata* have been investigated (Part IIa).

2. At harvest the initial composition of the Gros Michel fruit, grown in Central America, varies with the season of the year during which development occurs (Part IIc). 'Summer-grown' fruit may be characterized as fruit which is relatively high in amide (both asparagine and glutamine) and relatively low in free histidine, whereas 'winter-grown' fruit at harvest is relatively higher in free histidine, lower in total amide, and is especially poor in glutamine.

3. Using the cultivar Gros Michel, the outstanding change during ripening (Part IIa) of late summer fruit (i.e. high glutamine–low histidine fruit) was a pronounced shift in the relative proportions of histidine (which increased) to total amide (which decreased); the decrease in amide was mainly in glutamine; and while this occurred, the proportion of soluble to protein nitrogen remained relatively constant. (In the metabolism of histidine in other organisms, actual transfers of nitrogen from glutamine to the imidazole group of histidine are known.) As histidine increases during ripening, the other amino-acids, which *in toto* represent about as much nitrogen as histidine, also became more prominent, especially serine, the leucines, and pipecolic acid (Fig. 1).

4. 'Winter-grown' fruit of Gros Michel, also from Central America, already high in histidine and low in glutamine, did not show this change from glutamine amide nitrogen to histidine during ripening, for this had already occurred during the longer period of its development. Thus the changes in the soluble nitrogen of the fruit, especially those involving the amide and histidine in Gros Michel, seem to be separable from the carbohydrate and pigment changes which occur during ripening, because in the 'winter-grown' fruit, which develops more slowly, these changes may occur prior to harvest and while the fruit is still green.

5. The most prominent keto-acid of Gros Michel fruit is pyruvic acid

(α-ketoglutaric acid occurs only at a very much lower level), and the keto-acid which is convertable to valine (α-keto-isovaleric) was unexpectedly prominent. The pyruvic-acid content declined sharply during ripening, and this was paralleled by a similar decrease in alanine.

6. Fruit of the variety Gros Michel, from other areas of cultivation and after different periods and treatment during shipment, had quite different composition—being low in glutamine, high in asparagine, and histidine was much less prominent, but arginine was also present. During the ripening of this fruit, amide (asparagine) nitrogen decreased, and the amino-acids *in toto* (especially arginine, leucine, and valine) tended to increase (Fig. 4 of Part IIB).

7. Analysis of the soluble-nitrogen fraction of the fruit shows that its composition is determined much more by such conditions as the region of cultivation and the conditions which obtain during development than by the variety (Part IIB). Gros Michel fruit, grown at different seasons of the year and in different places, may differ more in its nitrogen compounds than fruit from different cultivars (Gros Michel, Lacatan, Cavendish). The extreme predominance of total amide (asparagine and glutamine) and of histidine, over all the other amino-acids collectively, seems to be a characteristic of the Gros Michel as grown in Central America, and particularly so when it develops in the summer. By contrast, the same variety from other regions (British Cameroons) initially emphasized the amino-acids generally (40 per cent. of the soluble nitrogen was in amino-acids other than asparagine, glutamine, or histidine), and these increased during ripening till they (about 10 amino-acids) together represented 60 per cent. of the soluble nitrogen.

In one case of Lacatan fruit, the ripening pattern was reminiscent of a given sample of Gros Michel with an initially high-amide (asparagine, low-glutamine), low-histidine fruit, for it increased in histidine and amino-acids somewhat generally, while asparagine decreased during ripening.

Therefore, the problem of the variables that determine the nitrogenous composition of the banana fruit is concerned with the conditions that obtain during growth, harvest, storage, and ripening even more than with such differences as those that undoubtedly do obtain among the cultivars Gros Michel, Lacatan, and Cavendish.

8. The amino-acid composition of the total protein in the banana fruit remains relatively constant over conditions which cause the soluble fraction to vary greatly. Nevertheless some trends can be seen: 'high-glutamine, high-histidine' Gros Michel fruit from Central America had lower arginine content in its protein and a higher content of dicarboxylic acids (which in point of fact may have been present in the protein, at least in part, as their amides). Within the relatively narrow observed range of variation in the amino-acid composition of the protein during ripening, some consistent trends were evident even in different varieties. Serine, glycine, and threonine collectively increased, alanine increased, the dicarboxylic acids decreased, and proline decreased in prominence in the protein. This may mean that a certain amount of protein turn-over occurs during ripening, thus permitting a

progressive replacement of protein even though the total content remains but little changed.

9. Two main effects on the soluble nitrogen of the developing banana fruit have been discerned.

(*a*) In the first effect, the amount of nitrogen distributed between the two amides and histidine (approximately 70 per cent. from Gros Michel in Central America) is variously partitioned among these three substances at the time of normal harvest. This ranges from 60 per cent. of the soluble nitrogen being present as amide (25 per cent. as glutamine) and 8 per cent. of the soluble nitrogen as histidine (for the 'summer-grown' fruit) to 35 per cent. of the soluble nitrogen as amide (7 per cent. as glutamine), and 35 per cent. as histidine in the 'winter-grown' fruit. If the high histidine content of this fruit has developed prior to harvest, as in the slower-developing winter fruit, it does not subsequently change much during ripening. However, if, as in the more rapidly developing summer fruit, the histidine content has not risen to its maximum value prior to harvest, it may do so in preference to amide (mainly glutamine) during the ripening process. Thus the developing fruit first accumulates amide, then turns over its soluble nitrogen increasingly to histidine, while glutamine relative to asparagine declines (see Table 7 and Figs. 1 and 2).

(*b*) In the second effect, which occurs during development, the amount and relative composition of the soluble nitrogen change markedly through the ontogeny of the fruit.

(i) Some 30 days prior to the emergence of the inflorescence, the fruit may be regarded as concluding its phase of active cell division and its content of soluble nitrogen is then high. Through Sachs's 'grand period of growth' (that is up to 20 to 50 days after emergence, depending on the season), growth depletes the soluble-nitrogen reserves of the fruit faster than they are replenished, but, thereafter, storage soluble nitrogen again accumulates to the level which is attained at harvest.

(ii) The progressive predominance of asparagine in the fruit as development proceeds indicates that this substance is increasingly associated with storage as overall synthesis and growth subside. The predominance of glutamine and alanine in the soluble nitrogen of the very young fruit associates these substances more than asparagine with synthesis, although, as nitrogen is stored again in the maturing fruit, glutamine as well as asparagine accumulates.

(iii) The trends in the soluble-nitrogen content of 'winter-grown' fruit reflect the slower growth of that season, and the specific qualities of the fruit developing in this season of the year are no doubt a response to temperature conditions, although this has not yet been investigated.

(iv) While the low total nitrogen of the banana peel reflects the same general trend throughout development as that of the pulp, its composition is quite different, for histidine is virtually absent and γ-aminobutyric acid accounts for 70 per cent. of the soluble nitrogen.

10. While more work will need to be done on selected aspects of the nitrogen metabolism of the banana fruit, these data show that the nitrogenous composition of the fruit responds to many factors during the course of its development and post-harvest behaviour. Certain ontogenetic trends can be recognized which merge into the post-harvest ripening sequence, but, in the case of the nitrogen compounds, they may be somewhat independent of the colour and carbohydrate changes that accompany the ripening of the fruit. For reasons not yet known, the fruit grown in the Honduras has a metabolism involving greater emphasis on free histidine and on certain reciprocal changes with amide (particularly glutamine), but this is not now seen as a peculiarity of the cultivar Gros Michel, but rather as a reflection of environmental conditions, or nutritional practices. While differences between the nitrogenous composition of the fruit and its ripening behaviour have been seen and investigated in fruits belonging to different cultivars, these now seem to indicate the range of metabolic behaviour which the banana fruit can encompass rather than varietal differences *per se*.

11. Although some work has been done on the nitrogen metabolism of the apple fruit during its ontogeny, which is here recognized (McKee and Urbach, 1953), this is complicated by the development of the seed, and it has not yet paid as much attention to the behaviour of the individual nitrogenous metabolites as the present investigation. Consequently, the relations between the two types of investigation will not be discussed here.

12. Some studies of the distribution of nitrogen compounds within the fruit pulp emphasize the great differences that obtain between the inner placental region, with its vestigial ovules, and the surrounding fleshy pericarp (endocarp). The inner region composed of placentae is very much richer in total soluble-nitrogen compounds: this extends to all the commonly occurring substances, but the major difference is that both amides (asparagine and glutamine) are virtually absent from the endocarp, and pipecolic acid and histidine are more prominent in the endocarp of the ripe fruit (Stage 8). By inference, the amide–histidine conversion during ripening may be located more in the fleshy pericarp than in the loculus which accumulates soluble nitrogen that would otherwise be absorbed by the developing embryo.

Acknowledgements

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Physiological Investigations on the Banana Plant

III. Factors which Affect the Nitrogen Compounds of the Leaves

BY

S. R. FREIBERG[1] AND F. C. STEWARD

(*Department of Botany, Cornell University, Ithaca, New York*)

Abstract

Banana plants (*Musa acuminata* cv. Gros Michel) have been grown in sand culture, irrigated with a full nutrient solution and with solutions deficient in each of several nutrient elements. The growth was continued long enough to establish the conditions characteristic of these nutrient treatments. Extracts of leaves, sampled according to their position on the axis, have been made from plants grown in full nutrient solution; these extracts have been examined by chromatographic methods to detect and determine the various nitrogen compounds they contain (A) The relative proportions of the soluble nitrogen compounds of the banana leaf are quite different from those of the fruit and, in response to the deficiency of specified mineral nutrients, both the total amount and the relative composition of the soluble nitrogen fraction are greatly affected. The results are interpreted in terms of the more active accumulation of soluble compounds in young leaves and the maintenance of a low amide (glutamine) level in leaves engaged in protein synthesis. The accumulation of soluble nitrogen compounds when growth and synthesis are arrested, and the relative accumulation of a specified number of nitrogenous substances, due to the lack of a nutrient element, indicates that metabolic blocks in reaction pathways occur. Examples of these effects are given and are related to nutritional deficiency (B).

Contents

As leaves are formed progressively higher on the axis of the banana plant, polymorphic changes are observed. In the first place the shape of the successive leaves alters as the shoot grows. Also, leaves higher on the axis attain a larger size. In order to reveal the effect of the position on the axis on the soluble nitrogen compounds of the leaf and to determine how this composition may be altered by nutritional imbalance is the subject of this third paper of the series.

Experimental Material and Methods of Analysis

Leaves were obtained from Gros Michel banana plants which were grown in sand culture in greenhouses in Central America. The plants were initiated

[1] Present address: United Fruit Company, Central Research Laboratories, Upland Road, Norwood, Mass.

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from small pieces of banana rhizomes, each of which contained one vegetative bud. Containers of pure quartz sand were used, and the plants were irrigated with a standard Shive and Robbins full nutrient solution (Shive and Robbins, 1951), and in the outcome the plants grew quite uniformly. The nutrient solution was allowed to drain through the sand, was collected and recirculated at least once a day in the early stages of the growth. When the plants were small the nutrient solution was completely changed weekly; but as they grew larger the nutrient solution was changed twice a week. Excess salts that might otherwise have accumulated in the sand were removed weekly with distilled water. Growth was continued in this manner for $8\frac{1}{2}$ months, when leaves at different positions on the axis were sampled for analysis.

The latest leaf to uncoil from the otherwise enclosed apex, and which had reached its full expansion, was designated No. 1. Thereafter the successive leaves were numbered in basipetal succession along the axis. All leaf material for analysis was weighed and then placed in 80 per cent. ethyl alcohol. In this manner it was transported from Central America to Cornell University, where it was extracted according to the procedures described in the previous papers.

The free amino-acids were determined quantitatively according to standard procedures in this laboratory and as reported in the second paper of this series. The ureido compounds were qualitatively determined by the techniques described in the first paper.

A. *Effects Due to Age and Position on the Axis*

Leaves numbered 2, 5, and 9 were selected for analysis. These three leaves all represented normal, healthy, green leaves, and the differences in their size at maximum expansion are indicated by their fresh weight, as follows: leaf No. 2 weighed 291 g.; leaf No. 5 weighed 171·5 g.; and leaf No. 9 weighed 100 g.

The differences in the composition of these three sets of leaves with respect to their free amino-acids are shown in Table 1.

It will be evident immediately that the free amino-acid composition of the leaf is quite different from that of the developing fruit (see Part II). It has already been shown that the compounds which dominate the composition of the fruit are the amides asparagine and glutamine and the basic amino-acids arginine and histidine. These substances are of relatively minor importance in the soluble nitrogen fraction of the leaves, in which glutamic acid, alanine, and γ-aminobutyric acid are the predominating constituents.

Referring now to the typical soluble nitrogen content of the leaf it is clear that the youngest leaf, which is the highest on the axis, has much the greater soluble nitrogen content. In fact, for the leaves compared *at their full expansion* the soluble nitrogen content per unit fresh weight varied over a six-fold range. The obvious suggestion is that the younger, more active, leaves which have recently expanded deplete the leaves lower on the axis of their soluble nitrogen compounds.

The relative composition of the soluble nitrogen fraction is subject to

certain evident trends when one passes from the youngest expanded leaf on the axis to leaves which are much older. These trends are as follows: glutamic acid declines from a high value at the most recently expanded leaf to a much lower value in the older ones, whereas alanine and γ-aminobutyric acid show a reversed trend from a lower value at the younger leaf to a higher value in the older leaf. This, however, should not imply that there is an

TABLE 1

Free Amino-acid Composition of Banana Leaves at Different Stages of Development on Plants (Gros Michel) Grown in Sand Culture on Full Nutrient Solution

Amino-acid	Stage of development: 2nd youngest leaf	5th leaf from apex	9th and oldest erect leaf
Aspartic acid	3·0%	2·3%	3·4%
Glutamic acid	24·1	11·8	8·1
Serine	5·0	3·9	6·0
Glycine	1·2	2·9	1·7
Asparagine	8·7	9·1	Trace
Threonine	4·9	4·6	3·9
Alanine	18·4	21·9	31·6
Glutamine	0·6	1·3	1·7
Lysine	2·4	2·5	—
Arginine	Trace	0·8	—
Proline	6·0	8·8	Trace
Valine	3·8	8·0	5·6
Leucine(s)	3·2	2·6	0·4
Phenylalanine	1·6	—	—
Tyrosine	1·6	1·8	—
γ-Aminobutyric acid	15·5	17·7	37·6
Total μg. N per g. fresh weight	135·2	76·8	23·4

(Nitrogen present as each amino-acid as a percentage of the total soluble nitrogen determined by ninhydrin.)

absolute gain of soluble nitrogen *per leaf* in the older ones, for if the amounts are calculated as quantity per leaf then even alanine, which increased markedly as a percentage of the soluble nitrogen in the leaf, declined to approximately one-tenth on the basis of the *amount per leaf*: the same was also true of γ-aminobutyric acid.

B. *Effects Due to Nutritional Imbalance*

(i) *Effects on Growth and Symptoms of Deficiency*

To determine the broad effect of certain of the major mineral nutrients on the composition of the leaves, the nutrient solutions were modified with respect to the content of calcium, potassium, phosphorus, and magnesium. One month after the young banana plants had been grown in sand irrigated with water—a period which allowed the reserve tissue to be depleted of its existing nutrients—they were placed upon a modified nutrient solution as

follows. To demonstrate the effects of calcium deficiency the calcium in the nutrient solution was replaced by sodium, potassium was replaced by sodium and calcium, magnesium was replaced by potassium and sodium, and to demonstrate phosphate deficiency phosphate was replaced by sulphate. The other ionic constituents of the solution remained unchanged, and the trace element additives were the same throughout, and included iron, boron, zinc, manganese, and molybdenum. After $7\frac{1}{2}$ months of these treatments, by which time the plants in the control, full nutrient culture, had the composition indicated in Table 1, the plants were harvested for analysis.

In even the most drastically deficient nutrient solution the banana shoot apex grew sufficiently to lay down a succession of leaves, but the deficiency symptoms were shown, not only by the appearance of the leaves, but by the very drastic effects on the size of the plants in question. This can be seen from the data of Table 2 below.

TABLE 2

The Effects of Nutritional Imbalance on the Growth of Banana (Gros Michel) During $8\frac{1}{2}$ months in Sand Culture

	Complete Nutrient	−K	−Ca[1]	−Mg	−P
Height in inches .	76	33	41	24	10
Fresh weight of leaves in grams					
2nd youngest leaf .	291	81	137	15	18·8
5th leaf from apex .	171	82	—	19[3]	10·5[4]
9th leaf from apex .	100	39[2]	61	—	—

[1] The amount of growth in the calcium deficient solution may reflect some supply in the concrete containers even though they were painted with horticultural asphalt.
[2] Leaf No. 6.
[3] Average value from leaves Nos. 4 and 6.
[4] Average value from leaves Nos. 5 and 6.

For example, the plants varied in height in response to mineral supply over a 7·5-fold range, and the size of leaf numbered 2 varied over a twenty-fold range. A general description of the principal visible symptoms of nutrient imbalance that were noted is given below. For illustrations see Murray, 1959.

Potassium deficiency. The first symptoms of imbalance appear as brown to purplish-brown flecks along the edges and on the upper surface of the petiole. Somewhat later, older leaves exhibit a pronounced marginal yellowing or chlorosis which has an irregular pattern of penetration toward the leaf midrib. These chlorotic areas gradually become necrotic and marginal scorching results. The irregular pattern of this scorching distinguishes this deficiency from the uniform, well-defined marginal chlorosis and necrosis observed in calcium deficiency. At later stages the irregularly necrotic, potassium-deficient leaves become partially collapsed and the midrib recurved so that the tip of the leaf is pointed toward the base of the plant. The curved appearance of entirely brown and desiccated, erect lower leaves is characteristic of

potassium-deficient banana plants which are also stunted in contrast to plants grown on full nutrient solution.

Calcium deficiency. Symptoms of imbalance first appear as a marginal chlorosis of older leaves which is not more than 1 inch wide at its widest point. This chlorosis is followed by the appearance of necrotic lesions and eventual necrosis of the entire leaf margin. The leaf thus appears green with a brown and dried margin.

Magnesium deficiency. A marked interveinal chlorosis extending finally to the youngest leaves characterizes this type of imbalance. Plants are also stunted in height.

Phosphorus deficiency. A very pronounced stunting of the plants is associated with phosphorus imbalance. The distances between leaves on the pseudostem are very much shortened so that a 'rosette' appearance results, similar to that observed in nitrogen-deficient banana plants. Phosphorus-deficient leaves, however, have a dark-green colour in contrast to the chlorotic appearance on nitrogen-deficient leaves.

(ii) *Effects on the Soluble Nitrogenous Compounds of Leaves*

The metabolic implications of the nutritional imbalance were assessed by reference to their effects upon the soluble nitrogen compounds of the leaves; and the leaves analysed had a location on the axis as closely similar as possible to those of the normal leaves numbered 2, 5, and 9, for which the composition is described in the above table. The data which resulted from these analyses are shown in Table 3 for the amino-acids and in Table 4 for ureido compounds.

The principal biochemical effects that were observed can be summarized as follows in relation to each nutrient that was deficient.

Potassium. A lack of potassium is reflected in a gain of total soluble nitrogen and of total amino-acid, which becomes more apparent as the symptoms of deficiency become more extreme. At leaf No. 2 when the soluble nitrogen content is high the effect of the deficiency of potassium was not evident on the total soluble nitrogen, though it was quite apparent in the content of glutamine which increased from a very low level to 14 per cent. of the nitrogen. However, at leaf No. 5 when, in the normal case, the total soluble nitrogen would have fallen to considerably lower level than at leaf No. 2, this trend was reversed by the potassium deficiency which had caused the total soluble nitrogen to increase more than fourfold by leaf No. 6. This latter increase was conspicuous in terms of glutamine, which rose to as much as 24·2 per cent. of the total soluble nitrogen, and also in the case of asparagine which reached a level of 20 per cent. of the total soluble nitrogen. The dicarboxylic acids, mainly glutamic acid, also increased from 14 per cent. to over 25 per cent. in the potassium-deficient leaf. Among the amino-acids which were found to decline in their content in the soluble nitrogen fraction were proline and γ-aminobutyric acid. Pipecolic acid on the other hand, which is normally absent in the leaf, reached a substantial concentration (186 μg. of the acid per g. of the potassium-deficient leaf No. 6).

TABLE 3

Amino-acid Composition of the Alcohol-soluble Fraction of Banana Leaves Grown under Conditions of Nutritional Imbalance

(Each compound is expressed as per cent. of the total amino-acid nitrogen as determined by ninhydrin.)

Amino-acid		Complete 2*	−K 2	−Mg 2	−P 2	−Ca 2	Complete 5	−K 5	−K 6	−Mg 4–6	−P 5–6	Complete 9	−Ca 9
Aspartic acid	(2)	3·0	4·2	10·1	11·4	2·7	2·3	1·9	6·0	16·8	12·4	3·4	4·7
Glutamic acid	(3)	24·1	24·3	17·1	39·4	34·6	11·8	10·5	19·7	26·2	21·3	8·1	14·0
Serine	(4)	5·0	3·8	3·8	4·3	4·9	3·9	6·9	5·7	2·7	2·7	6·0	2·0
Glycine	(5)	1·2	0·6	0·4	0·8	1·7	2·9	1·6	0·3	trace	trace	1·7	10·7
Asparagine	(6)	8·7	6·2	5·2	5·5	7·3	9·1	15·2	20·5	—	2·7	trace	—
Threonine	(7)	4·9	4·0	4·7	4·2	4·8	4·6	4·9	5·9	3·5	8·8	3·9	trace
Alanine	(8)	18·4	15·6	9·7	8·9	13·8	21·9	9·7	3·5	11·5	9·6	31·6	21·7
Glutamine	(9)	0·6	14·3	10·2	6·8	3·9	1·3	24·2	20·6	1·3	11·8	1·7	—
Lysine	(12)	2·4	—	—	—	1·5	2·5	2·1	1·0	—	—	—	—
Arginine	(13)	trace	—	—	—	5·3	0·8	1·2	—	—	4·1	—	—
Proline	(15)	6·0	8·7	5·1	8·9	1·3	8·8	0·2	0·2	6·6	12·0	trace	—
Valine	(16)	3·8	3·1	5·0	2·1	3·0	8·0	5·3	3·4	4·9	0·5	5·6	1·3
Leucine	(18)	3·2	2·1	3·5	0·4	1·6	2·6	4·6	1·2	4·2	5·6	0·4	trace
Phenylalanine	(19)	1·6	—	—	—	—	—	trace	1·2	—	—	—	—
Tyrosine	(21)	1·6	—	—	—	0·7	1·8	—	—	—	—	—	—
γ-Aminobutyric acid	(23)	15·5	13·1	7·5	5·8	12·9	17·7	9·9	4·8	9·1	6·6	37·6	45·6
Pipecolic acid	(26)	—	—	17·7	1·0	—	—	1·8	6·0	13·2	1·9	—	—
Homoserine	(27)	—	—	—	0·5	—	—	—	—	—	—	—	—
		100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0
Total micrograms alcohol soluble amino-acid nitrogen per g. fresh tissue as determined by ninhydrin		135·2	107·1	94·4	101·0	150·1	76·8	180·7	329·0	75·6	98·7	23·4	30·0

* Number refers to position of leaf on plant, counting the youngest leaf as No. 1.

TABLE 4

Ureido Compounds of the Alcohol-soluble Fraction of Banana Leaves Grown when Conditions of Nutritional Imbalance Detected with Ehrlich's Reagent Sprayed on Phenol: Acid-Butanol Chromatograms

Compound	Complete 2*	−K 2	−Mg 2	−P 2	−Ca 2	Complete 5	−K 5	−K 6	−Mg 4–6	−P 5–6	Complete 9	−Ca 9
Allantoic acid	xx	xxx	x	xx	x	xx	—	—	xx	x	—	—
Allantoin	—	—	—	—	—	—	xx	xx	—	—	—	—
Citrulline	xx	x	—	xxxx	—	—	xx	xx	—	xxx	—	—
Urea	xx	xxx	—	—	—	—	xx	xx	xx	xx	—	xx

* Number refers to position of leaf on plant, counting the youngest leaf as No. 1.

x = Trace; xx = Present; xxx = Prominent; xxxx = Very Prominent.

Severe nutritional imbalance due to lack of potassium brings about the accumulation of allantoin in banana leaves (see Fig. 4). Neither healthy leaves nor leaves lacking any of the other elements were found to contain allantoin. Concomitant with the appearance of allantoin, allantoic acid disappeared from these severely potassium-deficient leaves.

Before summarizing the data of Tables 3 and 4 on the effects of potassium on the identifiable soluble nitrogen compounds, certain other facts should be mentioned. Potassium deficiency in the banana leaf was notable because it was associated with the appearance on chromatograms of a relatively large number of nitrogenous compounds which have not, as yet, been identified. These compounds could be revealed by both ninhydrin and by Ehrlich's reagent, and their location on the chromatogram has been reported in Part I of this series. If the nitrogen equivalent of all these compounds had been evaluated, they would have increased markedly the figures for soluble nitrogen as shown in the table. An unusual compound was detected in potassium-deficient banana leaves in the form of putrescine. This volatile amine was first detected by Richards and Coleman (1952) in potassium-deficient barley plants.

It was therefore not unexpected to find that the main effects of potassium deficiency in the leaf could be explained in the following general way. Assuming that glutamine is one of the most immediate precursors of protein synthesis, so much so that it is maintained at a very low level in the full nutrient leaf, the immediate effect of potassium deficiency could be attributed to an impairment of protein synthesis, leading promptly to an accumulation of glutamine which is evident even before visible symptoms occur in the leaf. Thereafter, as the potassium deficiency becomes more extreme, glutamine accumulation occurs, and is *followed by* an accumulation of asparagine which could be attributed to protein breakdown. No doubt the effects of potassium deficiency ultimately extend to almost all areas of the intermediary metabolism, which accounts for the very large number of unidentified substances which appear on the chromatograms and react with ninhydrin and Ehrlich's reagent.

Magnesium. Effects of magnesium deficiency on the soluble nitrogen fraction are quite different from the effects of potassium deficiency. In this case the effects are not evident on the total amount of soluble nitrogen present in the leaves, but rather on the complement of amino-acids which make up this fraction. The most evident and quite unexpected effect of magnesium deficiency is the appearance of pipecolic acid which is not present appreciably in the full nutrient leaf, but in the magnesium-deficient leaf becomes the dominant substance, accounting for up to 17 per cent. of the total soluble nitrogen. It is interesting that this is accompanied by the virtual disappearance of lysine, since it has been shown that pipecolic acid commonly arises by ring closure from lysine. This implies the preferential storage of nitrogen as pipecolic acid rather than as lysine in the magnesium-deficient banana leaf. Among the amino-acids which became less prominent in the

soluble nitrogen of magnesium-deficient banana leaves were the following: alanine, asparagine, γ-aminobutyric acid. Among the amino-acids which became more prominent in the soluble nitrogen of the magnesium-deficient banana leaves were the dicarboxylic acids and pipecolic acid. In passing, it is of interest that Possingham (1956) found pipecolic acid to increase in manganese- and iron-deficient tomato plants. It is now known to accumulate under a variety of conditions (Pleshkov and Fowden, 1959; Yatsu and Boynton, 1959; Freney, Delwiche, and Johnson, 1959).

Phosphorus. Phosphorus deficiency causes the almost entire suppression of elongation growth and of the enlargement of the leaves, the effects on the soluble nitrogen compounds are therefore noted in terms of the relative changes within a total soluble nitrogen fraction which does not change appreciably in total amount. The amino-acids which increase in their percentage contribution to the soluble nitrogen are the dicarboxylic acids, glutamine, arginine, threonine, and homoserine. (Homoserine appeared under this nutritional situation although it does not occur normally in the banana leaf.) By contrast, the amino-acids which became less prominent under conditions of phosphorus deficiency are asparagine, alanine, and γ-aminobutyric acid.

The relative accumulation of glutamine and arginine with phosphorus deficiency might be attributed to the almost total lack of protein synthesis; and the simultaneous increase in aspartic acid, homoserine, and threonine is compatible with relationships between these amino-acids which have been suggested for legumes (Rabson and Tolbert, 1957). Arginine accumulation has previously been noted to occur in minerally deficient plants as follows. Arginine accumulation is a prominent feature of sulphur deficiency which has been observed by Coleman in white clover, tomato, and flax (1957); by Mertz and Matsumoto in alfalfa (1956); by Holley and Cain in iron deficiency of blueberry (1955); and arginine has been shown to accumulate prominently in sulphur-deficient mint plants in this laboratory (Crane, 1951; see also Steward and Pollard, 1956). Arginine accumulation due to phosphorus deficiency in alfalfa, and various effects of mineral deficiency on the nitrogen compounds of mint and tomato are described by Geleiter and Parker (1957) and Steward *et al.* (1959). Among the less familiar nitrogen compounds, which appear prominently in the phosphorus-deficient banana leaves, is the substance citrulline (see Part I, Fig. 4), which has also been seen to occur in response to nutrient imbalance in other situations (Coleman, 1957). The accumulation of arginine and citrulline in phosphorus-deficient leaves, with the disappearance of urea where citrulline was most prominent, suggests the importance of this element in an ornithine-citrulline-arginine-urea cycle.

Calcium. In the case of calcium the effects of the deficiency on the size and composition of the soluble amino-acid pool are relatively small. The main effect seems to be that the amide glutamine accumulated in the younger calcium-deficient leaves, though it tended to disappear from the older, whereas the corresponding dicarboxylic glutamic acid increased progressively with age.

Summary and Conclusions

(1) The first evident point is that the composition of the leaves with respect to the nitrogen compounds is quite different from the composition of the fruit, for the amino-acids which are prominent in the latter (namely asparagine, glutamine, arginine, and histidine) are far less prominent in the composition of the soluble nitrogen of the normal leaves. By contrast, the amino-acids which are most important in the leaves, namely the dicarboxylic acids alanine and γ-aminobutyric acid, are less prominent in the developing fruit. Even so, however, the pool of soluble nitrogen compounds in the leaf is markedly affected by the way it develops, as shown by the fact that the fully expanded leaves present on the axis at any one time have different composition by virtue of their position. Other examples showing that older leaves have less total free amino-acids are cited by Pleshkov and Fowden, 1959.

It is recognized, however, that the comparisons made on the composition of the leaves grown on full nutrient solutions have been made only at one season of the year and under greenhouse conditions. Therefore, it is still possible that the composition of the leaf, like the composition of the developing fruit, might be subject to variation in response to environmental conditions which are associated with the season of the year. It has been well demonstrated in other work done in this laboratory (Crane, 1951) with the mint plant (*Mentha piperita*) that the soluble nitrogen fraction is markedly responsive to both length of day and to day and night temperature (Howe, 1956; Rabson, 1956).

(2) Although it has not yet been possible to assess the importance of the environmental factors on the composition of the banana leaf, the results of nutrient imbalance are clearly evident, the effects being especially prominent under deficiencies of potassium, magnesium, and phosphorus. Whereas the normal trend in the banana leaf is toward a decreased soluble nitrogen content in leaves that are lower on the axis, suggesting export of soluble nitrogen to more active leaves above, the superimposed effect of mineral deficiency is markedly to reverse this trend, especially in the case of potassium. This suggests that in the potassium-deficient leaves protein synthesis is arrested, protein breakdown is enhanced, and that this is associated with the accumulation first of glutamine and later of asparagine.

(3) In all the nutritional deficiencies yet investigated on the banana plant the lack of the essential nutrient is associated with the accumulation of certain metabolites, suggesting that in response to the deficiency certain metabolic blocks exist. Obvious examples of these are the pronounced accumulation of pipecolic acid, associated with a decrease of lysine, in magnesium-deficient leaves; an accumulation of dicarboxylic acid in almost every deficiency reported; the relative accumulation of arginine and citrulline in the phosphorus-deficient plants, and the occurrence of homoserine, whereas it does not occur in the normal plant; the relative accumulation of

allantoin with a decrease of allantoic acid in severely potassium-deficient plants, which also form putrescine. Gamma-aminobutyric acid invariably becomes less prominent in response to nutritional imbalance.

(4) Therefore, sufficient has now been reported to show the marked effects of (*a*) development and (*b*) nutrition on the nitrogenous composition of banana leaves. By further intensive investigations of this kind, these effects could be more specifically localized in the metabolism and the effects due to other nutrient elements described.

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